Protection of Normal Tissues with 2-Aminoethylisothiouronium during Local Pelvic Radiation in Monkeys

Clara M. Ambrus, Julian L. Ambrus, Moshe Friedman, John W. Pickren, Edwin A. Mirand, and Stephen P. Kishel


ABSTRACT

Intestinal and bladder injury are the main limiting factors to radiation therapy in patients with pelvic neoplasms. 2-Aminoethylisothiouronium (AET) is a radiation-protective agent when given systemically but absorbs poorly from the intestines. Accordingly, it was explored for the local protection of the bowel and bladder during radiation to the pelvis. Radiation localized to the pelvis in various high fractionated doses and various schedules was applied to pairs of stumptailed monkeys (Macaca arctoides): one was always a control; and the other was treated with AET. AET was applied to the bladder through a catheter and to the rectum with a cotton tampon during the time of radiation. After radiation, AET was removed by repeated washings. Control animals developed hemorrhage, diarrhea, and emaciation and died at various times after completion of the radiation course; biopsy of rectal mucosa showed severe radiation damage. AET-treated animals had only occult blood in the stools and suffered slight weight loss; rectal biopsies showed normal tissues 2 weeks after radiation.

INTRODUCTION

Radiotherapy is considered to be an important modality in the treatment of carcinoma of the cervix, vagina, prostate, colon, rectum, and Stage B to D bladder cancers. The sites of pelvic cancers and the locations of most of the metastatic lymph nodes are encompassed by the boundaries of the bony pelvis. Curative radiation doses delivered to the organs may damage normal structures within the field of radiation. The most sensitive tissues are colon, rectum, and small bowel; the second most sensitive one is the bladder. Radiation sensitivity of these organs represents the most important limiting factors to curative radiation therapy. Radiation damage was reduced substantially by the time-dose modifications of radiation delivery (12, 18, 21, 23, 25, 27, 30, 31). Protection of normal tissues from radiation damage is still a challenge in spite of a long-standing search for effective chemical agents or the means to reduce tissue oxygenation (1, 6-9, 32).

In previous studies (5), solutions of 2-mercaptoethylguanidine hydrobromide prepared from AET* were used to perfuse the intestinal tract of Fischer 344 rats during exposure to whole-body radiation. This procedure was shown to extend significantly the lethal dose of whole-body radiation when combined with bone marrow transplantation. The most significant finding was protection of the intestinal tract from radiation damage. Therefore, it was thought to explore the radiation-protective effect of AET solution in primates when applied locally to the rectum and bladder during pelvic radiation. AET is one of the most active radiation-protective agents the activity of which is based chiefly on the availability of sulfhydryl groups (17, 22). Its toxicity prevents systemic administration (15, 16, 28); however, this can be bypassed with short-term local application.

In the following report, we describe the protective effect of AET on the normal structures of the rectum and bladder during pelvic radiation.

MATERIALS AND METHODS

AET Solution. A solution of AET (Schwartz Co., New York, N. Y.) was prepared by dissolving 250 mg in 100 ml of 0.15 M phosphate buffer at pH 10.5, the pH of the resulting solution being brought to pH 7.0 by titration with 0.1 N sodium hydroxide. This procedure rapidly converts AET to the active form, 2-mercaptoethylguanidine hydrobromide. The solution was always prepared immediately before radiation was to be used.

Monkeys. The study was performed on female stumptailed monkeys (Macaca arctoides) of 3 to 5 kg body weight. The animals were maintained on Purina monkey chow supplemented with oranges, apples, and bananas. Animals were maintained in compliance with standards as established by NIH and set forth in the Guide for the Care and Use of Laboratory Animals (34).

Preradiation Treatment. Two weeks before experiments started, the monkeys were put on weekly Bicillin (Wyeth Laboratories, Philadelphia, Pa.); 300,000 units i.m., to be continued until 1 month after the last dose of radiation.

One day before radiation, the animals were fasted and given water ad libitum with 5 teaspoons of sugar added to each bottle as a mild cathartic and food source.

Procedures on the Day of Radiation. Animals were restrained with ketamine hydrochloride (Ketaset; 15 mg/kg; Bristol Laboratories, Syracuse, N. Y. i.m. supplemented as needed; then, the animal was weighed. A Fleet phosphate enema (Fleet Pharmaceuticals, Lynchburg, Va.) of 130 ml was administered and was followed 2 hr later with washing of the rectum using 200 ml 0.9% NaCl solution.

Immediately before radiation, the rectal and pubic areas of the animal were washed with soap and water; the urethral area was washed with an aqueous solution of Zephran (1:750). The monkey was draped with sterile towels leaving the pubic area exposed.

Using sterile techniques, a urethral catheter (Intramedic Tubing No. 7440; 0.055 inside diameter x 0.075 outside diameter) was inserted into the urethral opening and into the bladder. The bladder was drained, and the urine was collected for examination in a sterile tube either through a syringe or by gravity drainage. Thereafter, 50 ml AET solution were passed through a Swinnex-25 filter unit (SXHA 2505, 0.45 µm; Millipore Corp., Bedford, Mass.) for sterilization and was introduced into the bladder through the catheter.

A sanitary cotton tampon soaked in AET solution was inserted full-length into the rectum using a lubricated analscope after the rectum was washed with 0.9% NaCl solution and freed of fecal material.

Control animals underwent the same procedure, using 0.9% NaCl solution instead of AET solution.

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2 To whom requests for reprints should be addressed.
3 The abbreviation used is: AET, 2-aminoethylisothiouronium.

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For the time of radiation, the animals were immobilized on a wooden board lying on their backs with the pelvis over an open port.

To ascertain that the rectum and the bladder were filled properly, a 0.9% NaCl solution containing 10% of a contrast medium (Renografin 60; E. R. Squibb & Sons, Princeton, N. J.) was used initially to soak the rectal tampon and fill the bladder; filling was checked using an X-ray picture. This checking procedure was later abandoned when sufficient experience was accumulated.

After radiation, the tampon was removed, and the rectum was flushed with 200 ml of 0.9% NaCl solution. The bladder was flushed similarly with 0.9% NaCl solution with repeated filling and emptying through the catheter. After these procedures, the monkeys were returned to their cages and put in upright positions to prevent aspiration. Food and water were replaced. If the animal lost a great deal of weight and there was evidence of dehydration (on the basis of hematology, blood chemistry values, general appearance, and behavior), before returning the animals to the cage, an infusion of 50 to 100 ml lactate Ringer injection USP (Travenol Laboratories, Deerfield, Ill.) was given i.v. depending on the animal's weight and degree of dehydration.

**Radiation.** Radiation was delivered with a 6 million-V linear accelerator, and the dose was divided between 2 opposing fields. Anteroposterior and posteroanterior doses were calculated midline of the pelvis. The limits of the fields were at the obturator fossa laterally 1 cm from the pelvic bone and superior to the level of L 5. Various dose schedules used are described in "Results."

**Rectal Biopsy.** Biopsies were taken from the rectal mucosa at the time when 1000 rads had been delivered and 2 weeks after completion of the radiation course. The removed tissue was placed in a buffered formalin and processed for histopathological examination.

**Laboratory Tests.** Feces removed from the rectum were examined for overt or occult blood. Urine removed at the time of catheterization was examined for hematuria, proteinuria, glucose, and pH. Blood samples for hematological (hematocrit, hemoglobin, quantitative, and qualitative blood counts) and blood chemistry (electrolytes, blood urea nitrogen, glucose, and acetate) evaluation were removed weekly, as soon as the Ketamine took effect, before the preradiation procedures were initiated.

**RESULTS**

In the first part of the study, we explored various pelvic radiation dose schedules to find one that initially produced a graded tissue response detectable in biopsy specimens and, after full-dose delivery, produced death in the untreated animals. Our previous experience (2-4) concerned toxicity of whole-body radiation to monkeys. Intensive search of the literature and inquiry at several primate centers revealed that there is no threshold dose in individual structures is not known. Table 1 summarizes our experiences.

Pelvic doses of up to 5000 rads given according to a schedule of 1000 rads weekly (in one session) did not produce significant damage. Apparently, the animals were able to recover in 1 week from the acute effect of the individual radiation doses. Occult blood was present in the feces from the second week of radiation until 2 weeks following radiation. Some diarrhea developed in the fifth week. There were no changes in hematological parameters or electrolytes at any time. We did not observe any skin changes. Periodic biopsies of the rectal mucosa did not show significant radiation damage except for some hyperemia of submucosal vessels and some inflammatory infiltrates. There was no ulceration at any time.

When 7000 rads were delivered in 5 weeks, giving 2 increments weekly of 700 rads each, occult blood was present from the second week on; this changed to overt bleeding on the fourth week, but no diarrhea developed. There were no changes in hematological parameters or electrolytes at any time. Periodic biopsy of the rectal mucosa did not show significant radiation damage; certainly none in proportion to the bloody stools.

Definite radiation effect could be established when the overall time of radiation was reduced to 3 weeks, and the total dose delivered to the pelvis was 6000 rads in 6 increments given twice weekly at 1000 rads/increment. After 2 weeks of radiation treatment, bloody diarrhea developed, and the monkeys showed signs of dehydration as evidenced from hematological parameters. The bloody diarrhea disappeared 1 week after radiation was discontinued. Periodic rectal biopsies indicated radiation damage including ulceration, congestion, and inflammatory infiltration of the mucosa which appeared by the third week of radiation. At 1 to 2 weeks after completion of the radiation course, the control animals died.

When the radiation dose was aimed at 9000 rads with biweekly increments of 1125 rads, death ensued shortly after the accumulated dose reached 8750 rads. Autopsy showed typical acute radiation damage by gross pathology and by microscopic study.

On the basis of these results, the pelvic radiation dose of 6000 rads delivered in 3 weeks was chosen for experiments testing the protective effect of AET. In 3 separate experiments, 6 animals were used, always in pairs, one control and one AET-treated, simultaneously. Rectal biopsies were taken at the end of weeks 1, 3, and 5 or at autopsy.

Table 2 summarizes the results. In control animals, the clinical course of radiation response was as described above: steady loss of weight; development of bloody diarrhea; dehydration; and death within 2 weeks after completion of radiation course.

In AET-treated monkeys, the weight loss was much less than that in the controls; occult blood appeared in the stools, but no overt bleeding or diarrhea developed. Two weeks after radiation, the animals were in good condition. Chart 1 shows changes in body weight, hematocrit, hemoglobin, WBC, and blood urea nitrogen in the control and AET-treated animals during the 3-week course of radiation. In the control animals, these changes

<table>
<thead>
<tr>
<th>Radiation</th>
<th>Rectal bleeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total dose (rads)</td>
<td>Dose (rads) schedule</td>
</tr>
<tr>
<td>-----------</td>
<td>----------------</td>
</tr>
<tr>
<td>5000</td>
<td>1000/wk (5 wk)</td>
</tr>
<tr>
<td>7000</td>
<td>2 × 700/wk (5 wk)</td>
</tr>
<tr>
<td>6000</td>
<td>2 × 1000/wk (3 wk)</td>
</tr>
<tr>
<td>6750</td>
<td>2 × 1125/wk (3 wk)</td>
</tr>
</tbody>
</table>

Table 1: Effect of various doses of local pelvic radiation in stumptailed monkeys (Macaca arctoides)
Radiation Protection with AET in Monkeys

Table 2
Comparison of radiation response in 3 control and 3 AET-treated stumptailed monkeys

<table>
<thead>
<tr>
<th>Radiation interval</th>
<th>Treatment</th>
<th>Diarrhea</th>
<th>Rectal bleeding</th>
<th>Examination</th>
<th>Tissue changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000 rads</td>
<td>0.9% NaCl solution</td>
<td>None</td>
<td>Occult</td>
<td>Rectal biopsy</td>
<td>Ulceration of mucosa, dilated capillaries, venules, edema of serosa, accentuation of mesothelial cells Normal</td>
</tr>
<tr>
<td></td>
<td>AET</td>
<td>None</td>
<td>None</td>
<td>Rectal biopsy</td>
<td>Complete destruction of mucosa, replacement by exudate formed from inflammatory cells and bacteria, dilated capillaries in submucosa, thrombi in venules</td>
</tr>
<tr>
<td>6000 rads</td>
<td>0.9% NaCl solution ±</td>
<td>Overt</td>
<td>Occult</td>
<td>Autopsy</td>
<td>Same as after 6000 rads</td>
</tr>
<tr>
<td></td>
<td>AET</td>
<td>None</td>
<td>Occult</td>
<td>Rectal biopsy</td>
<td>Minimal changes, essentially normal</td>
</tr>
<tr>
<td>2 wks following 6000 rads</td>
<td>0.9% NaCl Solution ±</td>
<td>Occult</td>
<td>Autopsy Rectum Bladder Bone marrow in radiated area</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AET</td>
<td>None</td>
<td>None</td>
<td>Rectal biopsy</td>
<td>Normal</td>
</tr>
</tbody>
</table>

Chart 1. Changes in body weight and various hematological values during fractionated radiation delivering 2 × 1000 rads/week and up to total of 6000 rads in 3 weeks. Left, control monkeys: x, 95; C, 102; O, 109; •, mean. Right, AET-treated monkeys: x, 96; C, 104; O, 110; •, mean. × 150.

are very likely to be the result of diarrhea-induced dehydration. In the AET-treated monkeys, fewer changes are observed, and they are more difficult to explain in the absence of obvious fluid loss (Table 2). Occasional hydration of the monkey, as described in "Materials and Methods," is not altering evaluations, since it favors survival of the controls.

Biopsies taken at various time periods showed gradually increasing damage in the tissues obtained from control monkeys: (a) after 2000 rads, there was ulceration of the mucosa, dilation of capillaries, venules, edema of the serosa, and accentuation of mesothelial cells; (b) after 6000 rads, the mucosa was completely destroyed and replaced by exudate formed from inflammatory cells and numerous bacteria. Capillaries in the submucosa were dilated, and several thrombi were present in venules; (c) at autopsy, 2 weeks after 6000 rads, the rectal mucosa showed the same damage as was shown immediately after completion of radiation, the bladder showed signs of inflammation, and the bone marrow in the radiated area was hypoplastic (in the non-radiated area, hyperplasia of the granulocytic series could be observed). In the skin, dilation of capillaries and infiltration of inflammatory cells around parafollicles could be seen. The epidermis was intact.

In AET-treated animals, biopsies taken at the same time intervals as in the controls, and at the time when the controls had already succumbed, showed essentially a normal rectal mucosa with minimal changes indicating radiation effect. Photographs of representative histological sections from the rectal biopsies taken from control and AET-treated animals after 2000 rads (end of the first week of radiation) and after 6000 rads (end of third week of radiation) are shown in Figs. 1 to 4.

From the above experiments, it seems that local application of AET was able to protect the rectum and the bladder of monkeys from tissue injury and death produced by 6000 rads fractionated pelvic radiation. Cause of death in the controls was likely due to extensive intestinal damage resulting in bleeding and excessive fluid loss. Damage to the bone marrow could not have played a significant role, since only the marrow in the limited radiation field was exposed to radiation damage; in the rest of the body, bone marrow remained intact.

DISCUSSION

Protection of normal structures from radiation damage associated with radiotherapy of pelvic tumors has been a continuous challenge to oncologists. Several avenues have been explored: development of new equipment and devices for radiation delivery; variations in the fractionation of radiation dose schedules; application of radiation protective agents; and combinations of the above (13, 18, 19, 29). Most of the effort has been devoted to protection of the bowels, since the intestinal mucosa was found to be highly radiation-sensitive, and its damage has deleterious physiological consequences.

Derivatives of AET, "sulfhydryl protectors," were the chemicals most often tested alone for radioprotective effect or, to reduce O2 effect, in combination with vasoconstrictors and ATP. Toxicity of these chemicals by systemic administration has counterbalanced the benefits achieved by reduction of radiation dam-
age (10, 11, 20, 24, 26, 29, 33). We have selected AET for our study partly because of its poor absorption through the mucosal wall but mostly because of previous success with rodents in radiation protection of the gut with AET perfusion (5).

We have attempted to apply AET only temporarily, during the time of radiation, and to protect the bowel and bladder by direct contact of the chemical with the mucosa. To prove efficacy, we have used exaggerated radiation doses, producing not only morbidity but mortality in the control animals. The protective effect of AET against acute radiation damage was impressive. Extrapolation of results in experimental animals to human application is of course always difficult. Radiation doses applied to monkeys, when evaluating AET effect, were unusually high and were prohibitive in clinical situations. For example, in clinical radiation therapy, the most common time-dose relationship used is 200 rads daily given in 5 increments/week. This is approximately only one-half of the weekly dose given to the monkeys in the AET study, given in fewer increments because of the anesthesia and restraining necessary in the animals. Since AET proved to be protective even when uncommonly high radiation doses were used, it can be assumed that a moderate increase in radiation dose in the presence of AET will exert a higher tumoricidal effect, while the tolerance of normal tissue is preserved. However, this hypothesis will have to be substantiated in future experiments in pelvic tumor-bearing animals. Only then should clinical investigation be designed to explore the extension of therapeutic range afforded by AET protection when radiation is aimed at tumor size reduction and tumor eradication.

We have no information on the protective effect of AET against late but localized radiation damage. According to reports in the literature, AET did not afford long-range radiation protection against whole-body radiation of mice (25). The long-range consequences of pelvic radiation, however, consist mostly of localized defects (such as fistulas) and not generalized somatic damage. Accordingly, it is hopeful that localized protection by AET of intestinal and bladder mucosa may eliminate those chronic radiation effects.

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27. 

Fig. 1. Biopsy from AET-treated monkey after 2000-rad local pelvic radiation. Rectal mucosa is intact, and there is a mild increase of plasma cells in the lamina propria. Exfoliation of epithelial cells into the lumen and hyperplasia of columnar cells at the base of the crypts of Lieberkuhn have occurred. Submucosa is free of inflammatory cells. × 150.

Fig. 2. Biopsy from 0.9% NaCl solution-treated control monkey after 2000-rad local pelvic radiation. Mucosa is almost completely ulcerated with one dilated gland remaining. Capillaries are dilated in the mucosa. Surface is covered by colonies of bacteria. Gland is lined by cuboidal cells having enlarged hyperchromatic nuclei. Scattered inflammatory cells are present in the mucosa, and a few inflammatory cells are present in the submucosa. × 150.

Fig. 3. Biopsy of rectum of AET-treated monkey 2 weeks after completion of 6000 rads local pelvic radiation. Mucosa is intact. Some glands have lost mucus and secreting cells and have hyperchromatic nuclei, but none are dilated, and no inflammation is evident. × 150.

Fig. 4. Biopsy of rectum of 0.9% NaCl solution control monkey that died 2 weeks after completion of 6000 rads local pelvic radiation. Mucosa is ulcerated almost completely with one remaining dilated gland filled with an exudate of polymorphonuclear leukocytes. Perivascular infiltrations are present around blood vessels in the submucosa. Mucosa contains large numbers of polymorphonuclear leukocytes. × 150.
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