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Destruction of Implanted Gastric Tumors in Rats by Acridine Orange Photoactivation with an Argon Laser*

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Abstract—*The phototoxicity of acridine orange and argon laser irradiation on Walker carcinosarcoma 256 stomach tumors was studied. Wistar strain rats bearing stomach tumors 4–6 mm in diameter 5–10 days after their implantation were injected intraperitoneally with 40 mg/kg of acridine orange 2 hr before irradiation. Then the forestomach was opened and the tumors were exposed to the argon laser at 488 nm at an intensity of 15 mW/cm² for 20 min. Tumors in rats treated with acridine orange were brightly fluorescent during irradiation. No marked temperature rise was detected during irradiation. Argon irradiation significantly prolonged the survival of rats treated with acridine orange. Histologically, complete or partial tumor necrosis was observed, with sparing of surrounding mucosa, in rats treated by the combination therapy. Phase-contrast and electron microscopy showed that cytotoxicity was mediated by changes in the cell, nuclear and lysosomal membranes. Neither the dye nor laser alone had any effect.*

INTRODUCTION

PHOTOACTIVATION of biological materials or the action on them of certain light-activated dyes in the presence of oxygen has taken on new significance in recent years with the demonstration by several groups of its application in treatment of animal tumors [1–6]. Although Jesionek and Tappeiner [7] in 1903 claimed some success in treatment of skin cancer by the photodynamic action of eosin, no further studies were made on this subject until 1972, when Diamond *et al.* [1] showed that hematoporphyrin was effective as a photosensitizing dye in the treatment of animal tumors *in vivo*. Recently, Dougherty *et al.* [4] and Hayata *et al.* [8] reported success in the clinical treatment of human tumors by using hematoporphyrin derivatives and dye laser. In 1974 Tomson *et al.* [3] reported the use of acridine orange and argon laser in treatment of mouse epithelial tumors. In the present work the phototoxic effect of acridine orange with low-energy argon laser irradiation on implanted rat stomach tumors was investigated.

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MATERIALS AND METHODS

Walker stomach tumors

Tumor cells from a Walker 256 carcinosarcoma tumor were implanted into the gastric mucosa of Wistar strain rats as described by Brøyn [9]. A solid Walker tumor was excised from the subcutaneous space of a donor rat, minced and homogenized in saline solution, and the homogenate was passed through a gauze filter. The resulting suspension contained mainly free tumor cells. The cell concentration and the cell survival rate were counted in a Bürcker chamber after staining with 0.2% trypan blue in Hank's solution; unstained cells were regarded as viable. The concentration of tumor cells was kept at 5000–25,000/ml, of which 50–70% were viable. Before operation the rats were starved for 24 hr. Then their abdomen was opened under general anesthesia with ethyl ether and the stomach was lifted up. A vinyl tube was passed through the mouth to the stomach and 1 ml of tumor suspension was injected. Part of the gastric wall near the greater curvature was clamped from the outside with a special kind of

clamp, which was shut and then rapidly opened. Clamping of the stomach induced mucosal lesions, and the tumor cells were implanted into the gastric mucosa. Trauma was induced in the greater curvature of the gastric fundus. Then the tube was withdrawn during aspiration and the abdomen was closed.

Drug

Acridine orange was obtained from E. Merck AG (Darmstadt, F.R.G.) and used as a solution of 1 mg/0.1 ml of 0.9 %NaCl (adjusted to pH 1.8 with 0.5 N HCl) [5].

Treatment

Between 5 and 10 days after tumor implantation, tumor-bearing rats were randomly divided into 4 groups and treated as follows: group 1 was injected intraperitoneally with 40 mg/kg of acridine orange 2 hr before laser irradiation [5]. The abdomen was opened again under general anesthesia with ethyl ether. The stomach was lifted up and the forestomach was opened along the greater curvature. Then the gastric tumors were exposed to an argon laser at 488 nm at an intensity of 15 mW/cm^2 for 20 min. After irradiation the forestomach and then the abdomen were closed. Group 2 was injected with acridine orange in the same way as group 1. A midline incision was made in the abdomen and the forestomach was opened. Then the stomach was closed without irradiation with an argon laser. Group 3 was subjected to the operation and gastric tumors were irradiated with argon laser in the same way as group 1, but without pretreatment with acridine orange. Group 4 received no treatment except the gastric operation in the same way as group 1. In all groups the size of stomach tumors was measured at gastrotomy and rats bearing tumors of 4–6 mm in diameter were used for experiments. Histologically, these tumors had penetrated through the mucosal muscle layer but had not reached the serosal side [9]. Fifteen rats in group 1, 6 in group 2, 8 in group 3 and 10 in group 4 were killed on experimental day 7, and their stomachs were examined histologically to determine the response of the stomach tumors to treatment. The other rats were used to compare the effects of the different treatments on survival. A further 15 rats bearing tumors 6–10 mm in diameter were treated in the same way as group 1 and killed on experimental day 7 for histological examination of the depth of necrosis of stomach tumors caused by laser irradiation. Normal rats without stomach tumors were treated with acridine orange and then laser irradiation for 30 min in the same way as group 1. Five rats were killed 7 days after treatment to study the effects of the combination therapy on normal gastric mucosa by phase-contrast microscopy and for histological examination.

Laser irradiation

Light was delivered into a short optic fiber by coupling to the output beam of a Spectra Physics Argon Laser 171 (Spectra Physics, Inc., Mountain View, CA). The spectral output was centered at 488 nm, and the mean irradiation intensity was 15 mW/cm^2.

Surface temperature measurement

The surface temperatures of the gastric tumor and adjacent gastric mucosa were measured with a thermister thermometer (Terumo Medical Co., Ltd., Shizuoka, Japan).

Phase contrast and electron microscopy

Additional tumor-bearing rats were injected with acridine orange 2 hr before and killed 5, 10 and 20 min after laser irradiation in the way described above. Cytological changes of the gastric tumors were observed by phase-contrast microscopy [10]. Specimens were obtained by scraping implanted gastric tumors immediately after killing the rats, and observations were made with a Nikon SFCR-Ke Phase-Contrast Microscope (Nikon, Tokyo, Japan). Specimens obtained from tumors were also stained for acid-phosphatase activity by the method of Burstone [11]. Specimens obtained from gastric tumors treated with acridine orange were also observed by electron microscopy before and 10 and 20 min after laser irradiation. Pieces of gastric tumors were removed immediately after the rats were killed and were cut into small blocks, which were fixed in 2.5% glutaraldehyde, postfixed in 1.0% osmium tetroxide and embedded in epoxy Epon resin by routine methods. Each fixative was adjusted to pH 7.2 with 0.1 M cacodylate buffer, and fixation was carried out at 4°C for 60 min. Ultrathin sections were cut on an LKB Ultrotome and doubly stained with saturated uranylacetate and lead citrate. The sections were examined with an electron microscope, model JEM-100CX.

Statistical methods

Results were analyzed by Fisher's exact probability test [12]. Data on survival were evaluated by the method of Kaplan and Meier [13] and the generalized Wilcoxon test [14]. The word 'significant' indicates a calculated P value of less than 0.05.

RESULTS

Effects on stomach tumors

Tumors in rats treated with acridine orange showed intense fluorescence during laser irradia-

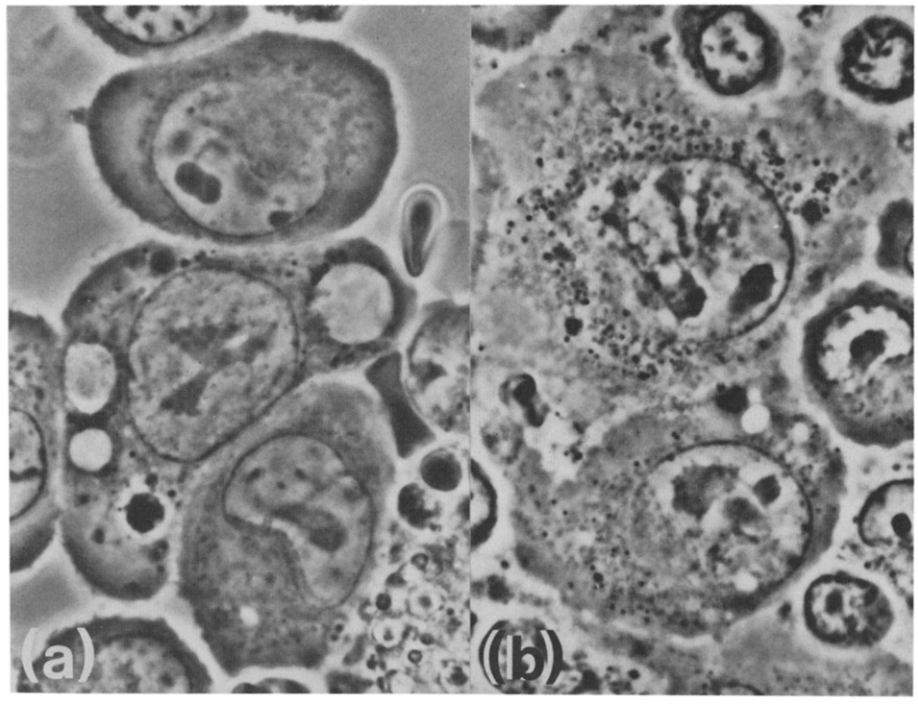

Fig. 1. Phase-contrast microphotograph of a Walker stomach tumor in a rat that received no treatment (A) and in a rat treated with acridine orange and laser irradiation for 20 min *(B). Laser irradiation caused marked nuclear pyknosis and blister formation.* ×1000.

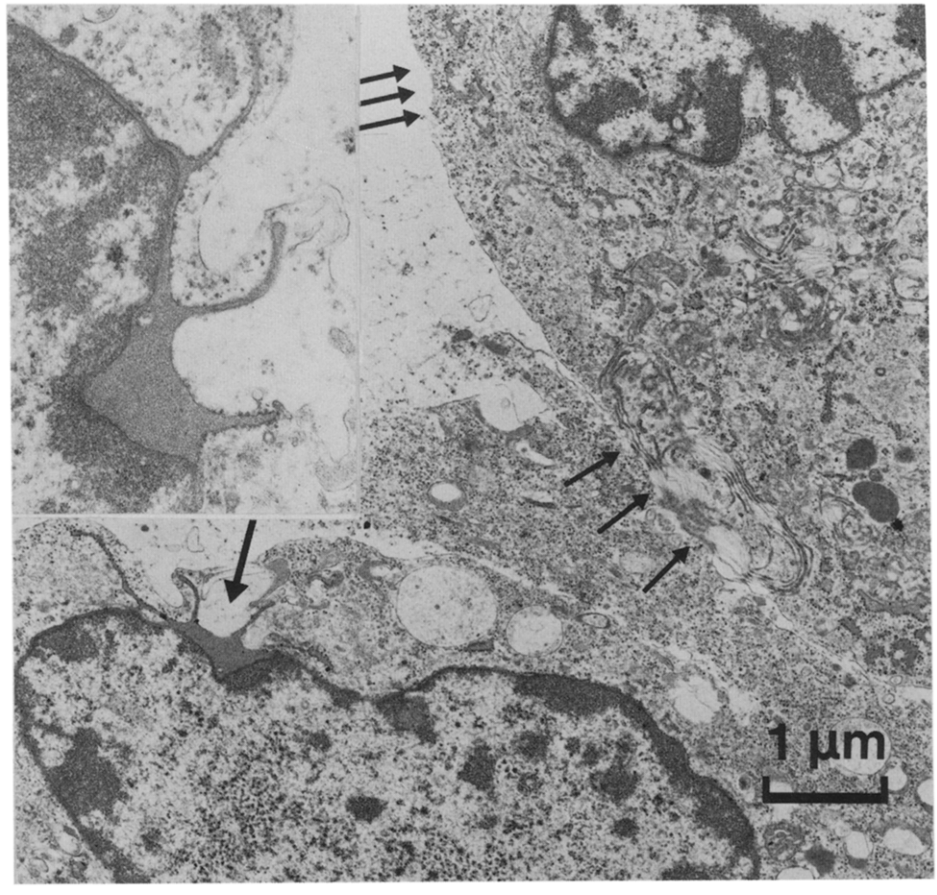

Fig. 2. (A) Electron microscopic findings in a Walker stomach tumor in a rat treated with acridine orange and then argon laser irradiation for 20 min. *The cell membrane had already disappeared (small arrows). The two layers of the nuclear envelope were separated by irregularly shaped gaps (large arrow). (B) Higher magnification showing that the karyoplasm was released into the nuclear envelope forming an expanded cavity with a dense matrix. N, nucleus. (A:* ×10,000; *B:* ×31,000).

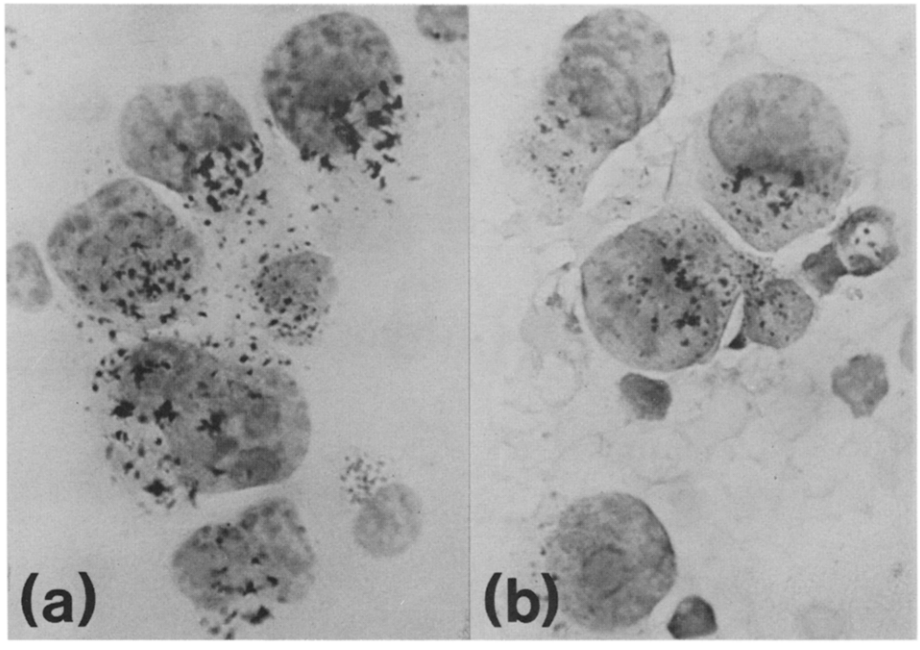

Fig. 3. Acid-phosphatase activity, seen as red granules, diffusely distributed in the cytoplasm of tumor cells in a rat treated with acridine orange, but not irradiated (A). Laser irradiation for 20 min resulted in decrease in the number and size of granules (B). (Azo-dye method, A: ×1000; B: ×1000).

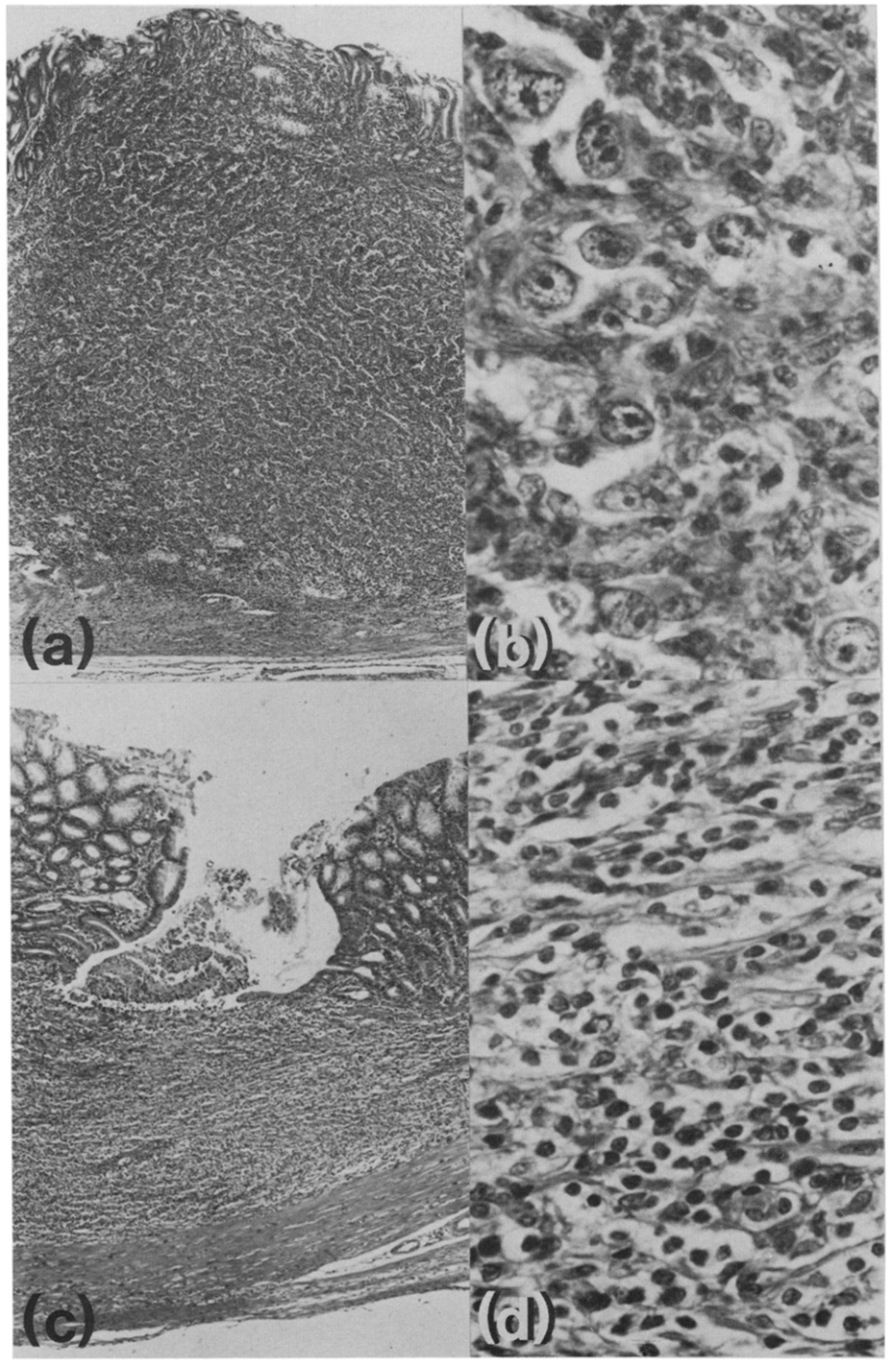

Fig. 4. *A Walker stomach tumor had already reached the serosal side* 7 days *after the experiment in control group* 4 (*A, B*). *However, complete tumor necrosis is seen in the irradiated area of a group* 1 *rat* 7 days *after treatment with acridine orange and then argon laser* (*C*). *Mucosal erosion is observed, but the surrounding gastric mucosa appears normal. Higher magnification* (*D*) *shows no tumor cells.* (*H and E, A*: ×25; *B*: ×200; *C*: ×25; *D*: ×200).

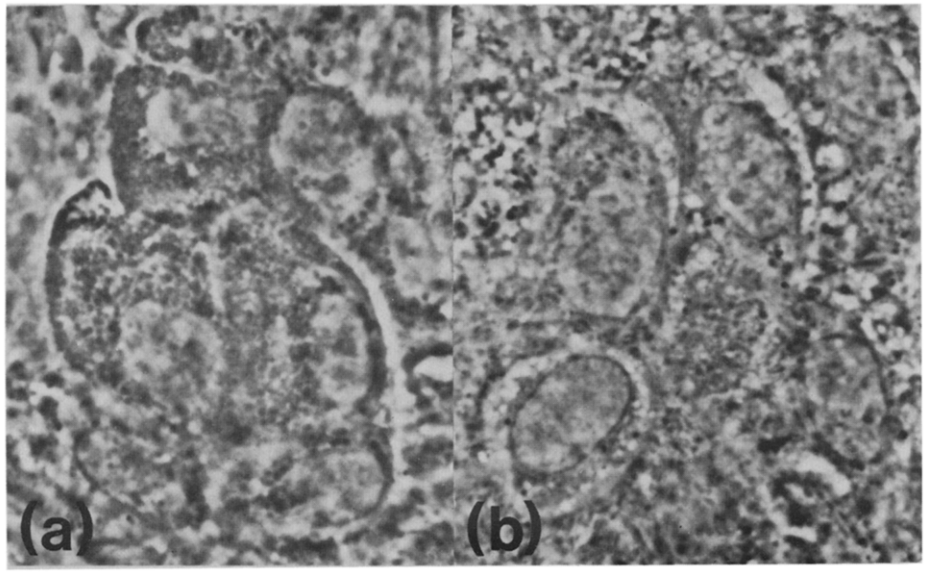

Fig. 6. Phase-contrast microphotographs of gastric mucosa in a normal rat treated with acridine orange alone (A) and in a normal rat treated with acridine orange and then laser irradiation (B).

tion. Measurement of the surface temperature of the gastric tumors and adjacent gastric mucosa showed that the temperature did not increase more than 3°C during irradiation.

By phase-contrast microscopy, the first signs of degeneration of the tumor were seen as early as 5 min after irradiation. Laser irradiation produced progressively more damage of the tumor cells. As shown in Fig. 1, 20 min after the beginning of irradiation the nucleus had become markedly pyknotic and the nuclear membranes had become thicker and irregular; dense chromatin aggregates were seen in the nucleus. Blisters developed rapidly, compressing numerous dense granules near the nucleus. Electron microscopy showed that at this time the cell membrane had already disappeared, that the two layers of nuclear envelope were separated by irregularly shaped gaps and that the karyoplasm had been released into the nuclear envelope, sometimes forming expanded cavities with a dense matrix (Fig. 2). Acid-phosphatase activity was seen as red granules in the cytoplasm of tumor cells. These granules were diffusely distributed in the cytoplasm of tumor cells treated with acridine orange but not irradiated. However, laser irradiation for 20 min resulted in a decrease in the number and size of granules, which remained chiefly near the nucleus (Fig. 3). Treatment with acridine orange without irradiation or laser irradiation without acridine orange treatment had little or no influence on tumor cells.

The histological changes of gastric tumors 7 days after acridine orange treatment and/or laser irradiation are summarized in Table 1. Tumor irradiation with argon laser caused extensive necrosis of all tumors in group 1 treated with acridine orange. Eight out of 15 rats had no microscopically detectable tumor cells but normal adjacent gastric mucosa (Fig. 4). The mean depth (± S.E.) of the necrosis of stomach tumors was 4.3 ± 0.3 mm (range 2.1–6.6 mm). Partial tumor necrosis was observed in 7 other rats in group 1. Acridine orange treatment without laser irradiation in group 2 and laser irradiation without acridine orange treatment in group 3 caused no detectable histological necrosis of tumors.

Figure 5 shows the survival of rats after different treatments. The mean tumor sizes (± S.E.) at the time of treatment in groups 1, 2, 3 and 4 were 4.9 ± 0.2, 4.8 ± 0.3, 5.0 ± 0.3 and 4.8 ± 0.3 mm respectively; the differences among these values were not statistically significant. All rats in control groups 2, 3 and 4 died from tumor extension within 8 days, but the survival of rats in group 1 after concomitant treatment with acridine orange and laser irradiation was significantly longer than that of controls in group 4; 4 out of 10 rats treated with acridine orange and then laser irrradiation were still alive 14 days after treatment.

Effects on normal gastric mucosa

Phase-contrast microscopy showed that treatment with acridine orange and then laser irradiation had little or no influence on normal gastric mucosa. Comparison with cells from normal gastric mucosa of rats treated with acridine orange alone showed that 30 min after the beginning of combination therapy the nuclear membrane was irregularly thickened and that numerous enlarged dull-gray granules had appeared in the cytoplasm (Fig. 6). Seven days after treatment 7 out of 12 specimens of the stomach showed no detectable histological changes of the gastric mucosa, one showed deep erosion with hyperemia, two showed superficial epithelial defects with mucosal hyperemia and one mucosal hyperemia only, but none showed ulcer formation.

DISCUSSION

The phototoxic effect of acridine compounds was first reported by Raab [15] in 1900 and was studied in detail early in this century by Tappeiner and Jodelbauer [16]. In 1974 Tomson *et al.* [3] reported that mouse tumors, including invasive squamous cell carcinoma, could be destroyed in animals fed acridine orange orally and then irradiated with an argon laser, which did

Table 1. *Response of Walker carcinosarcoma* 256 *implanted into the stomach to acridine orange and laser irradiation*

Group No. and treatment	Histological findings in gastric tumor: Complete necrosis	Partial necrosis	No evidence of necrosis	Total
1. Acridine orange + laser irradiation	8 (53.3%)	7 (46.7%)	0 (0.0%)	15
2. Acridine orange alone	0 (0.0%)	0 (0.0%)	6 (100%)	6
3. Laser irradiation alone	0 (0.0%)	0 (0.0%)	8 (100%)	8
4. No treatment	0 (0.0%)	0 (0.0%)	10 (100%)	10

The differences between the incidences of tumor necrosis in groups 1 and 2, in groups 1 and 3 and in groups 1 and 4 were statistically significant ($P < 0.001$, $P < 0.002$ and $P < 0.001$ respectively).

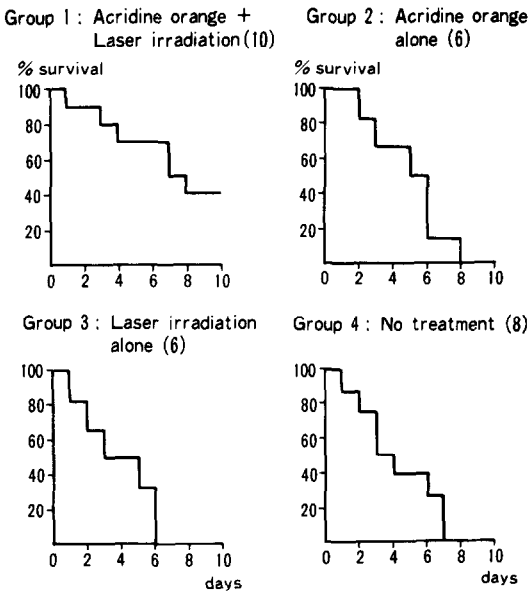

Fig. 5. Survival curves of rats bearing Walker stomach tumors. Survival of rats in group 1 was significantly ($P < 0.05$) longer than that of controls in group 4.

not cause either a significant increase in temperature or histological damage to normal skin or to the tissues surrounding the tumors. The present results indicate that argon laser irradiation at energy levels that did not cause heat damage significantly prolonged the survival of rats treated with acridine orange.

Concomitant treatment with acridine orange and laser irradiation caused complete or partial necrosis of implanted gastric tumors without damaging the surrounding gastric mucosa. Treatment with acridine orange without laser irradiation or argon laser irradiation without acridine orange did not have any effect.

Acridine orange has a strong affinity for intracellular DNA and RNA in living cells [17, 18]. In 1964 Sherif [19] reported that the cervix uteri can be examined for cancer by swabbing it with acridine orange solution and inspecting it with a beam of u.v. light, malignancy being correlated with a yellow fluorescence. Ackerman *et al.* [20, 21] reported that tumors implanted into the lung of rats showed selective uptake of aminoacridine compounds. These compounds were detected in lung tumors by examination under u.v. light. The aminoacridines were detected more clearly in the tumor tissue than in surrounding lung tissue, and after 24 hr only the tumor tissue fluoresced under u.v. light. This fluorescence of tumors was weak but still present after 7 days. Evidence for selective localization of aminoacridines in implanted Walker stomach tumors has also been obtained [21]. However, it is not yet known why aminoacridines are selective for tumors.

A 1-μM aqueous solution of acridine orange shows an absorption peak of 492 nm, and this dye forms at least 2 complexes in solution with both DNA and RNA, with absorption maxima at 465 and 502 nm [22]. The argon laser emits 9 spectral lines between 454.5 and 514.5 nm. We used an argon laser beam at 488 nm as a light source because it was the most powerful beam close to the maximum absorption band of acridine orange.

There are a few reports on the morphological effects of acridine orange and laser irradiation on tumor cells [23]. In the present study the first signs of degenerative changes appeared in tumor cells treated with acridine orange as early as 5 min after irradiation. Laser irradiation produced progressively more damage of the tumor cells; irradiation for 20 min caused marked nuclear pyknosis, disappearance of cell membranes, alteration of the nuclear membrane and decrease in the number of granules staining red with azo-dye [11]. The acid-phosphatase staining reaction demonstrates the presence of lysosomes, so it seems likely that lysosomes were destroyed by laser irradiation. Therefore marked alterations of the nucleus and cell and nuclear membranes, and release of catabolic enzymes from the lysosomes may cause tumor necrosis. Acridine orange alone and laser irradiation alone had little or no influence on the cytology of tumor cells.

This phototoxic system may prove useful as a method for detecting and treating gastric cancer. We have developed an endoscopic method using acridine orange and a mercury vapour arc lamp for diagnosis of gastric cancer [24]. In this method gastric cancer showed yellow fluorescence, in sharp contrast to the green fluorescence of unaffected gastric mucosa. However, several problems must be solved before this method can be used for treatment of human gastric cancer. One is that penetration of 488-nm light through the tissue is low. In this study the mean depth of necrosis after acridine orange treatment and then laser irradiation at 488 nm was 4.3 ± 0.3 mm. Similarly, Tomson [5] reported that the depth of penetration of 488-nm light was approximately 5 mm. However, he found that the depth of destruction of tumor tissue by this light was up to 9 mm and suggested that besides penetration of the light, there may be an additional effect due to release of lysomal enzymes into adjacent tumor cells. Recently, Kato [25] reported that the depth of the tumor affected by the concomitant use of a blue argon laser beam and hematoporphyrin derivatives was usually more than 15 mm. Dougherty *et al.* [26] reported that the depth of penetration of red light was usually 20 mm, whereas Hayata *et al.* [27] reported that the depth of destruction of the tumor by the combined use of

a red laser beam and hematoporphyrin derivatives was less than 5 mm.

In Japan, with the widespread use of endoscopic gastric biopsy and cytology under direct vision for diagnosis of gastric cancer, early gastric cancers are recognized with increasing frequency. When gastric cancers are confined to the gastric mucosa and are small in size, no distant metastasis is found. Moreover, the depth of gastric cancer can be determined exactly by endoscopy [28]. Therefore it seems possible that this phototoxic system could completely destroy minute superficial gastric cancers without the necessity for laparotomy.

The second problem in our method is the toxicity of acridine orange. Although acridine orange is toxic when given in excess [29], its oral administration at 10 mg/kg was sufficient to impart a fluorescence to gastric cancer tissue [24]. In our previous work, 4 out of 35 patients complained of nausea but none showed severe adverse effects. Recently, we found that intraperitoneal injection of 10 mg/kg of acridine orange was sufficient to cause remarkable degeneration of tumor cells in rats after laser irradiation at an irradiance of 15 mW/cm^2. Although acridine orange is mutagenic [29], Duuren *et al.* [30] concluded that it was not an initiator or a carcinogen in mouse skin. But they reported that it induced a small number of tumors in the liver and at the injection site in rats and mice. It is possible to inject acridine orange into a gastric tumor under direct vision with a gastrofiberscope, and we found that this was effective for destruction of the tumors. Local injection into a gastric tumor seems better than oral administration of acridine orange because a lower dose is effective.

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