Timing Effects of Combined Radioimmunotherapy and Radiotherapy on a Human Solid Tumor in Nude Mice[1]

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ABSTRACT

Timing effects of radioimmunotherapy (RIT) combined with external-beam radiotherapy (RT) were assessed in human colon carcinoma xenografts. Initially, dose effects of fractionated RT and RIT were evaluated separately. Then, 30 Gy RT (10 fractions over 12 days) were combined with three weekly i.v. injections of 200 μCi of ^{131}I-labeled anti-carcino-embryonic antigen monoclonal antibodies in four different treatment schedules. RIT was given either prior to, concurrently, immediately after, or 2 weeks after RT administration. The longest regrowth delay (RD) of 105 days was observed in mice treated by concurrent administration of RT and RIT, whereas the RDs of RT and RIT alone were 34 and 20 days, respectively. The three sequential combination treatments produced significantly shorter RDs ranging from 62 to 70 days. The tumor response represented by the minimal volume (MV) also showed that concurrent administration of RT and RIT gave the best result, with a mean MV of 4.5% as compared to MVs from 26 to 53% for the three sequential treatments. The results were confirmed in a second experiment, in which a RT of 40 Gy was combined with an identical RIT as above (three injections of 200 μCi of ^{131}I-labeled monoclonal antibodies). At comparable toxicity levels, the maximum tolerated RT or RIT alone gave shorter RDs and less tumor shrinkage compared to simultaneous RT+RIT. These results may be useful for designing clinical protocols of combined RIT and RT.

INTRODUCTION

Radiolabeled antibodies are currently evaluated for treatment of a variety of human malignancies. However, with the exception of lymphomas (1), there are few reports of successful clinical RIT[3] of solid tumors. Treatment of solid tumors with RIT has proved to be very difficult due to the small amounts of antibody that can be targeted to tumors and the BM depression associated with systemic administration of large quantities of radiolabeled antibody (2, 3). Consequently, new methods are required to improve the effectiveness of RIT (4, 5).

On the basis of preclinical and clinical experience of over a decade (6–8), an attractive scenario is that RIT or other antibody-mediated treatments could be considered as a boost to conventional treatments such as surgery, RT, or chemotherapy for elimination of minimal residual disease (9, 10).

Improved local tumor control by RT has been shown to have a significant impact on patient survival, especially in tumors of low metastatic potential (11). The efficacy of radiation treatment is dose dependent. Prescription habits in radiation oncology are based mainly on the tolerance of dose-limiting healthy tissues. On the other hand,

external-beam irradiation has only a local effect, and micrometastases distant from the radiation field will not be affected by this treatment. Therefore, combination of RT and RIT might be beneficial for local control and might offer a way for spatial cooperation as well. A combination of RIT and RT is expected to give a greater tumor response and patient survival than either of the components alone, with a level of overall toxicity that does not exceed that of either treatment alone. In addition, debulking by the first treatment means that fewer cells will have to be killed by the second one and may also lead to improved oxygen supply and, possibly, to an increased uptake of radiolabeled antibody under these conditions (12–14).

Following the pioneering work of Order et al. (15), RT combined with RIT or with other systemic radiation therapy has been proposed recently for clinical use (16, 17), but few experimental data exist (18, 19). In a previous study, we have demonstrated an additive therapeutic effect of combined RT and RIT, as measured by increased RD and local control (19). However, in that study, the tumor volumes were relatively small, and both RT and RIT were administered in a less clinically relevant way, i.e., in high doses per fraction. Furthermore, to our knowledge, the effect of the timing of the combined treatments of RT and RIT has never been addressed. Here we have investigated the time-dependent interaction of fractionated RIT and RT on larger human colon carcinoma xenografts and on normal tissues in a nude mouse model.

MATERIALS AND METHODS

Tumor Model. All experiments in nude mice were performed according to Swiss legislation and approved by the official committee on surveillance of animal experiments. Seven- to 9-week-old female Swiss homozygous *nu/nu* nude mice were given a s.c. transplantation, in the midline of the back at 2 cm from the tail, of a volume of about 30 mm³ of freshly excised, minced Co112 colon cancer (20). Three to 4 weeks after inoculation, the mice bearing tumors of approximately 60–120 mm³ volume with a mean tumor volume of about 90 mm³ were weighed for each of the test treatments.

Irradiation of Tumors. X-rays were generated by a Philips RT 250 operating at 200 kV and 20 mA. The beam was filtered with 0.5 mm Cu (half-value layer, 1 mm Cu). Irradiation of tumor transplants was as described previously (19). The dose rate in this setup was 0.64 Gy/min, with a dose heterogeneity of ±5.5% for an 8-mm tumor. To obtain dose homogeneity, the mice were rotated 180 degrees at alternate treatments with a regime of 10 fractions over 12 days (5 fractions per week as in the clinical situation).

MAbs. Four anti-CEA MAbs, MAbs 35, B7, B17, and B93 (21, 22), all of the IgG1 subclass, and one irrelevant control mouse IgG1 secreted by the mouse myeloma P3×63 were used. The four MAbs are directed against four independent epitopes of CEA (23) and were purified by ammonium sulfate precipitation and ion-exchange chromatography (21, 22).

Radiolabeling of MAbs. A pool of the four anti-CEA MAbs (at equal concentrations) was labeled with ^{131}I for therapy and for biodistribution studies (including an ^{125}I-labeled irrelevant control IgG1) using the Iodogen method. Radiolabeled protein was separated from free iodine by filtration on Sephadex G25 columns (Pharmacia). After filtration, >96% of radioactivity was protein bound in all preparations, giving a final specific activity of 3–6 μCi/μg protein. Immunoreactivity was determined for all labeled preparations according to the methods described previously (21), and an anti-CEA binding of

[3] The abbreviations used are: RIT, radioimmunotherapy; RT, radiotherapy; RD, regrowth delay; CEA, carcinoembryonic antigen; % ID/g, percentage of injected dose/g tissue; MAb, monoclonal antibody; MV, minimal volume; BM, bone marrow.

80.3 ± 4.5% was obtained. Nonspecific binding of radiolabeled anti-CEA MAbs to a control protein on Sepharose was 1.3 ± 0.7%.

RIT and Combined Therapies. To evaluate the optimal RIT protocol to use in the combination of RIT and RT, we have compared the tumor cytostatic effect and the toxicity of multiple administrations of ^{131}I-labeled MAbs with single administrations. In single-dose RITs, groups of nude mice bearing established Col12 xenografts were injected in the tail vein with 100, 200, 400, 600, or 800 μCi per mouse of ^{131}I-labeled anti-CEA MAbs. Multiple doses were given, with a 1-week interval between injections. Thus, an 800-μCi total dose per animal was distributed over eight injections of 100 μCi, four injections of 200 μCi, or two injections of 300 μCi plus one of 200 μCi of radiolabeled MAbs. For the combined therapy, three injections of 200 μCi were chosen, because a small additional BM toxicity was expected to occur due to RT.

Different therapeutic strategies have been evaluated for the combination of RIT and RT, as shown in Fig. 2. For the first group, RT (30 or 40 Gy in 10 fractions over 12 days, with 3 or 4 Gy/fraction/day, respectively) and RIT (three injections of 200 μCi of ^{131}I-labeled MAbs, with a 1-week interval between injections) were started at the same time; this group was designated RT+RIT. The second group of animals, designated RT-RIT, was treated by a full course of RT followed by RIT. The first injection of RIT was given on the last day of RT. For the third group, designated RT-2W-RIT, RT was given first followed after a delay of 2 weeks by RIT. For the fourth group, designated RIT-RT, RIT was given first followed by RT 3 days after the last ^{131}I-labeled MAb injection. All four combined therapy schedules were evaluated in two blocks, each block including half of the mice of each group with similar-sized tumors.

Experimental End Points. Tumor size was measured twice a week for the first 2 months and then once a week. The three diameters (d_1, length; d_2, width; and d_3, height) were measured with calipers, and the tumor volume (V) was calculated using the following formula: $V = [d_1 \times d_2 \times d_3/2]$. The relative tumor volume (V/Vo) was calculated by dividing the measured tumor volume (V) by the initial tumor volume (Vo) at day 0. The time required for tumor volume increase by 3 times the initial treatment size was calculated for each mouse, and the absolute tumor RD was obtained by subtraction of the mean RD in untreated mice (5.5 ± 1.8 days; $n = 10$). The MV was defined as the smallest tumor volume after treatment in percentage of tumor volume at day 0. The absence of palpable tumor mass 6 months after the end of treatment was taken as an indication of local control.

Toxicity Evaluation. Local skin toxicity of RT was evaluated by inspection three times per week for the first 5 weeks and then twice per week. The scores for skin toxicity in radiation field used were as follows: I, faint redness; II, partial necrosis; and III, complete necrosis. Toxicity following injections of radiolabeled MAbs or combined administration of RT and RIT was evaluated by weight measurements, inspection for petechiae, and counts of peripheral WBCs and platelets. Body weight was measured 3 times weekly from the first injection of radiolabeled antibodies until no radioactivity was detectable. Formation of petechiae was noted 3 times per week. Peripheral WBCs and platelets were monitored at least once 14 days after the last injection of radiolabeled MAbs (correlated with petechiae formation at that time). WBCs and platelets were counted according to the methods described previously (19). No late toxicity was observed in any mice treated with radiolabeled MAbs alone or with combined therapies.

Biodistribution Studies. Tumor-bearing nude mice were irradiated with 6, 15, and 30 Gy using 3 Gy/fraction/day. Mice were given injections (4 h after the last irradiation) of 200 μl of radiolabeled MAb mixture and control IgG i.v. (24). The injected dose per mouse was 2 μCi of ^{131}I-labeled MAbs and 2 μCi of ^{125}I-labeled irrelevant control IgG. The specific activity was 3 μCi/μg protein. To match the conditions of RIT, 50 μg of cold anti-CEA MAbs or 50 μg of irrelevant control IgG per mouse were injected together with the radiolabeled proteins. The nonirradiated tumor-bearing mice were dissected at different times after MAb injection. The irradiated mice were dissected 24 h after injection, using four mice per group. All mice were sacrificed by CO_2 inhalation; 0.5 ml blood was obtained; and the tumor, normal tissues, and carcasses were dissected and weighed, and the radioactivity for both iodine isotopes was measured in a dual-channel gamma scintillation counter. The antibody uptake in each tissue was expressed in terms of % ID/g (24).

Dosimetry. Calculations of radiation doses for tumor and blood were based on the time course study of ^{131}I tissue distribution in mice injected with trace

amounts of ^{131}I-labeled MAbs together with unlabeled antibodies. First, effective ^{131}I-labeled MAb retention in tissues was calculated from the amount of radioactivity measured directly at different time points. Then, an integral activity in μCi × h was calculated per g of tumor and blood (25). Tissue-absorbed β-radiation for tumor and blood was then calculated according to the following equations (26).

$$D_\beta = 2.13 \times \mu Ci/g \times h \times E_\beta \text{ rad}$$

$$E_\beta \text{ of } {}^{131}I = \frac{0.19 \text{ g}}{\mu Ci \times h}$$

Additional γ-radiation was assumed to be distributed equally in the whole animal. The γ-radiation represents about 10% of the β whole-body radiation for a 25–30-g mouse (26).

Histology. Tumors that were untreated or irradiated with 6, 15, and 30 Gy (3 Gy/fraction/day and 5 fractions/week) were removed from nude mice 24 h after the last irradiation and fixed in a solution of 1% paraformaldehyde-2% glutaraldehyde at 4°C. The specimens were embedded in methacrylate. Two-μm sections were cut and stained with Gill's H&E for microscopic examination.

Immunohistochemistry of Frozen Tissue Sections. To compare the CEA expression within tumors that were untreated or irradiated with 6, 15, and 30 Gy, tumor tissue was removed 24 h after the last irradiation, frozen in isopentane cooled by liquid nitrogen, and kept at −80°C until use. Nine-μm cryostat sections were incubated for 60 min with a mouse-human anti-CEA chimeric antibody IgG (10 μg/ml) (27) and then incubated for 60 min with an antihuman IgG antibody coupled to horseradish peroxidase (Dako, Glostrup, Denmark) diluted 1:80. Controls were performed with an anti-swine IgG antibody coupled to horseradish peroxidase. All incubations were performed at room temperature and followed by a wash with PBS. Peroxidase activity was revealed by adding a freshly prepared solution containing 3-amino-9-ethylcar-bazole (Sigma Chemical Co., St. Louis, MO) and 0.06% H_2O_2, and counter-staining was performed with Gill's hematoxylin.

Statistical Analysis. Relative tumor RD, MV, and hematological toxicity in the various conditions of therapy were evaluated using the Student-New-man-Keuls multigroup test.

RESULTS

RIT with Single and Repeated Injections of Radiolabeled MAbs. Tumor response and toxicity of RIT using a single dose of ^{131}I-labeled MAbs were dose dependent (Table 1). RIT with 100 and 200 μCi of ^{131}I-labeled MAbs only slightly inhibited tumor growth with minimal tumor RDs. RIT with 400 μCi of ^{131}I-labeled MAbs stopped tumor growth for only about 2 weeks. This dose corresponds to the maximal tolerated dose. RIT with 600 and 800 μCi of ^{131}I-labeled MAbs was highly toxic, leading to the death of four of eight and three of four mice, respectively (Table 1). Toxicity of RIT with 600 or 800 μCi manifested as weight loss, BM depression, and development of petechiae observed 10–17 days after therapy in most animals. Death in some of the animals (Table 1) also occurred during this time period. To evaluate the therapeutic potential of high-dose RIT, five additional mice were treated with 800 μCi of ^{131}I-labeled MAbs and 7 and 10 days later were transplanted with BM from untreated mice. RIT with 800 μCi resulted in marked tumor shrinkage and a tumor RD of about 3 or 4 weeks, but no cures were observed. These results demonstrate the relative radioresistance of the human tumor line Col12 used here, compared with the high percentage of local controls that we reported previously with similar doses of RIT of the human colon carcinoma T380 xenografts (22).

To obtain more efficient tumor killing with less toxic effects, radiolabeled MAbs were administered in several fractions at weekly intervals, as suggested by previously reported RIT results (28, 29). The dose fractions used were eight injections of 100 μCi, three

Table 1 *Tumor effect and host toxicity of RIT given as a single dose*

Dose of ^{131}I-labeled MAbs	No. of mice	RD (days)[a]	MV[b] (%)	No. of mice that died (%)
100 µCi	5	4.5 ± 4.3	100 ± 0.0[c]	0
200 µCi	5	4.2 ± 1.9	100 ± 0.0[c]	0
400 µCi	5	15.9 ± 5.2	100 ± 0.0[c]	0
600 µCi	8	24.3 ± 5.2[d]	67.5 ± 39.8[d]	4 (50)
800 µCi	4	(50)[e]	(21)[e]	3 (75)
800 µCi	5	27.1 ± 7.6[f]	69.8 ± 32.9[f]	0

[a] Mean absolute tumor RD (±SD).
[b] Mean tumor MV (±SD).
[c] No tumor shrinkage occurred in these mice.
[d] Mean RD or MV (±SD) from four surviving mice of eight.
[e] RD or MV from one surviving mouse out of four.
[f] Mean RD or MV (±SD) from five BM-rescued mice.

Table 2 *Tumor effect and host toxicity of RIT given in fractionated doses*

Dose of ^{131}I-labeled MAbs	No. of mice	RD[a] (days)	MV[b] (%)	No. of mice that died (%)
100 µCi eight times	6	nc[c]	100.0 ± 0.0[d]	0
200 µCi three times	7	20.3 ± 4.9	100.0 ± 0.0[d]	0
200 µCi four times	6	47.9 ± 9.2	82.0 ± 24.5	0
300 µCi two times + 200 µCi one time	6	53.3 ± 3.5[e]	19.9 ± 2.3[e]	3 (50)

[a] Mean absolute tumor RD (±SD).
[b] Mean tumor MV (±SD).
[c] nc, not calculated (RD could not be calculated following the definition).
[d] No tumor shrinkage occurred in these mice.
[e] Mean RD and MV (±SD) results from the three surviving mice out of six.

injections of 200 µCi, four injections of 200 µCi, and two injections of 300 µCi plus one injection of 200 µCi (Table 2).

In the group of six mice that received the 100-µCi fraction eight times, there was no tumor shrinkage and only moderate tumor growth inhibition in four mice and no effect on two mice. In the group that received 200 µCi four times, there was marked tumor RD (48 days) but moderate tumor shrinkage (MV, 82%), whereas in the group treated twice with 300 µCi plus once with 200 µCi of ^{131}I-labeled MAbs, significant tumor shrinkage occurred in all mice (MV, 20%).

Significant toxicity was observed in the group treated twice with 300 µCi and once with 200 µCi of ^{131}I-labeled MAbs. In this group, all mice had significant weight loss (>10%), five out of six mice developed petechiae between 1 and 7 days after the last injection, and three of them died. In the group treated with 200 µCi four times, weight loss was less important (9.3 ± 4.2%), and spontaneously reversible BM toxicity was observed (Table 3). Overall, the longest RD (48 days) obtained with fractionated RIT without severe toxicity was observed in mice treated four times with 200 µCi of ^{131}I-labeled MAbs (Table 2).

RT with Different Doses and Fractionation Schedules. The antitumor effect and skin toxicity of RT with 20, 30, 40, and 50 Gy in 10 fractions and of 50 Gy in 20 fractions over 12 days were evaluated (Fig. 1; Table 4). Skin toxicity was only observed in the group treated with 50 Gy. Nine out of 10 mice had skin toxicity grade III and 1 had grade II when 50 Gy were given in 10 fractions over 12 days, whereas 1 out of 5 mice had skin toxicity grade II and 3 had grade I when 50 Gy were given in 20 fractions over 12 days.

Tumor response was dose dependent for all radiation doses when expressed as tumor MV ($P < 0.05$), whereas, when expressed as RD, the differences between RT of 30 and 40 Gy were not significant.

An additional group of mice had RT of 30 Gy in 10 fractions given in 5 days. This accelerated RT gave much more tumor shrinkage than the same dose given in 12 days ($P < 0.01$), but the mean RD was similar between these 2 groups ($P > 0.05$; Table 4).

Combined Therapies of RT and RIT. Because RIT was found to be most efficient without severe toxicity when given in four doses of

200 µCi of ^{131}I-radiolabeled MAbs at weekly intervals, we decided to use a similar treatment for combined therapy. However, the number of weekly injections of 200 µCi was reduced to three to compensate for some BM irradiation during RT.

In a preliminary experiment of combined therapy, two groups of mice received a RT of 30 Gy in 10 fractions given over either 5 or 12 days and followed immediately by RIT of 200 µCi three times at weekly intervals. In the group that received 10 fractions over 5 days, all six mice had petechiae, and five of them died between 4 and 21 days after the last injection of ^{131}I-labeled MAbs. No petechiae or lethality was observed in the group treated with RT of 10 fractions over 12 days followed by RIT.

All combined therapies with 30 Gy RT in 10 fractions over 12 days and RIT of 200 µCi three times at weekly intervals produced a significantly longer tumor RD than the individual treatments of RT or RIT alone (see Fig. 3). Tumor responses however, were dependent on the sequence in which the two treatment modalities were given (Fig. 2). If RT and RIT were administered at the same time (RT+RIT), tumor RD was the longest, with a mean value of 105 days, for 13 out of 15 mice, and 2 mice had no relapse after 6 months (Fig. 3). This tumor RD of 105 days, as compared with that of RIT alone (20.3 days) and RT alone (34.1 days), suggests strongly that the simultaneous administration of RT+RIT produces a superior therapeutic effect as compared to the simple addition of RDs of the individual treatments ($P < 0.001$). The three sequential combined therapies produced similar tumor RDs ranging from 62 to 72 days, which were significantly shorter than the RD of the simultaneous RT+RIT ($P < 0.002$). Expression of tumor response in MV confirmed that concurrent administration of RIT and RT gave the best result, with a mean MV of 4.5% as compared to 25.8% for mice treated with RT immediately followed by RIT, 45.3% for RIT given before RT, and 52.5% for RIT given 2 weeks after RT. The tumor MV from mice that received simultaneous administration of RT and RIT was significantly smaller than that of all three sequential combined therapies ($P < 0.002$), whereas among the sequential treatments, the MV from mice treated with RT immediately followed by RIT was also significantly smaller than that of the other two sequential therapies ($P < 0.05$). In conclusion, concurrent administration of RT and RIT gave an overall maximum antitumor effect.

Concerning side effects, no skin toxicity and petechiae were observed in the four different combined therapy schedules, and the weight losses of less than 10% were not significantly different from those of the mice that received RIT alone (data not shown). Peripheral blood leukocytes and platelets of mice 14 days after the last RIT injection showed no significant differences in hematological toxicity

Table 3 *Peripheral blood analysis of mice at different days after the last injection of ^{131}I-labeled MAbs with dose fractionation[a]*

Time after last injection	200 µCi three times (n = 6)	200 µCi four times (n = 6)	300 µCi two times + 200 µCi one time (n = 6)
14 days			
WBC	1913 ± 954	540 ± 209	57 ± 24
Platelets (×10⁵)	9.3 ± 4.1	7.9 ± 2.1	1.4 ± 1.1
34 days			
WBC	ND[b]	2943 ± 690	1497 ± 379[c]
Platelets (×10⁵)		9.3 ± 2.4	5.2 ± 4.52[c]
56 days			
WBC	ND[b]	9150 ± 1850	6670 ± 2640[c]
Platelets (×10⁵)		13.7 ± 3.7	12.6 ± 3.8[c]

[a] The results are expressed in number/mm³ (mean ± SD). In comparison, the WBC count was 7406 ± 1280 and the platelet count was 13.8 ± 1.7 × 10⁵ in untreated control mice (n = 24).
[b] ND, not done.
[c] The results are from three surviving mice out of six.

among the four different combined therapy groups (Table 5). When compared with RIT (three treatments with 200 μCi of ^{131}I-labeled MAbs) alone, the groups of simultaneous RT+RIT and of RT followed immediately by RIT showed a significant further reduction of leukocytes ($P < 0.05$). Concerning counts of platelets, no significant difference was found either among the four different combined therapy groups or when compared to RIT alone.

A second experiment comparing the four combination schedules was performed using identical conditions except that the RT dose was increased to 40 Gy (4 Gy/fraction, with 10 fractions over 12 days). Compared to the first experiment, very similar results were observed in the four different combined therapy groups, except that more local tumor controls (5 out of 15 mice) were obtained in the simultaneous RT+RIT group (Fig. 4). Statistical evaluation of the second combined therapy experiment gave very similar results for MVs and RDs as in the first experiment. BM toxicity was not increased markedly and mice remained without petechiae, skin toxicity, and toxicity-related death. The two experiments therefore conclusively show that the simultaneous combination of RT and RIT is optimal as compared to the three other sequential treatments.

Comparison of RT and RIT with Combined Therapies. If we compare the antitumor effect of RIT and RT expressed as RD, fractionated RIT with three treatments of 200 μCi was equal to fractionated RT of 20 Gy (in 10 fraction over 12 days), whereas RIT
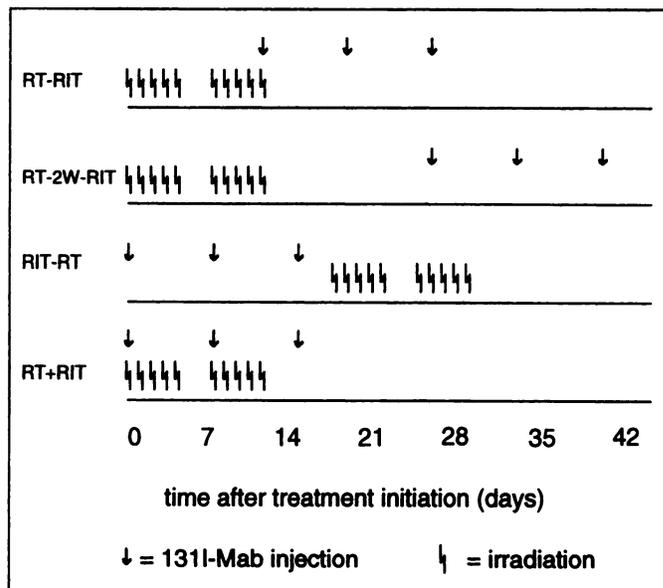
Fig. 2. The different time schedules of the combined treatments. *RT*, RT of 30 or 40 Gy administered in 10 fractions over 12 days; *RIT*, RIT of three injections of 200 μCi of ^{131}I-labeled MAbs each with a 1-week interval between injections; *RT-RIT*, RT followed by RIT without delay; *RT-2W-RIT*, RT followed by RIT with a 2-week delay; *RIT-RT*, RIT first followed by RT; *RT+RIT*, RT and RIT administered simultaneously.
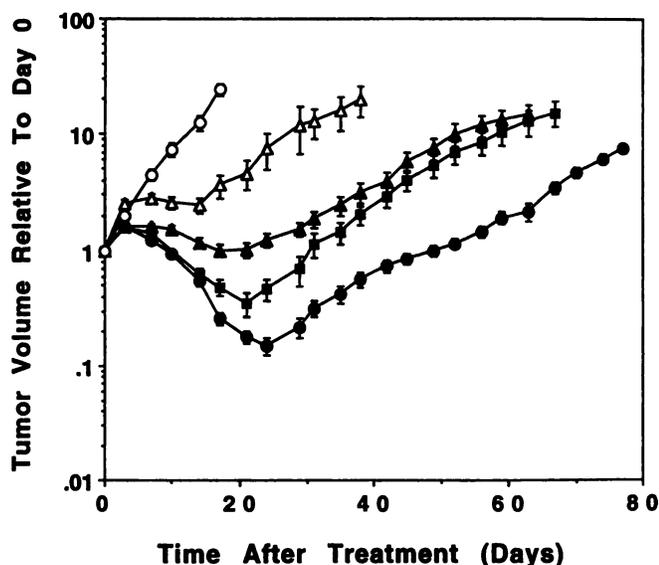
Fig. 1. Effect of RT of different total doses on growth of Col12 xenografts in nude mice. The mean tumor volume relative to the initial tumor volume at day 0 is plotted versus time for each group (±SE). RT was of 20, 30, 40, and 50 Gy each given in 10 fractions over 12 days. O, untreated control ($n = 10$); △, 20 Gy ($n = 6$); ▲, 30 Gy ($n = 9$); ■, 40 Gy ($n = 10$); ●, 50 Gy ($n = 10$); bars, SE.

with four treatments of 200 μCi was similar to RT of 40–50 Gy (in 20 fractions over 12 days). However, if the antitumor is expressed as MV, RIT with four treatments of 200 μCi was less efficient than RT of 40 ($P < 0.05$) or 50 Gy ($P < 0.01$). If we compare the optimal combination (simultaneous) of RT (30 Gy) and RIT (three treatments of 200 μCi) with the maximum tolerated RT alone (40 Gy, absence of skin toxicity) or the maximum tolerated RIT alone (four treatments of 200 μCi, absence of petechiae and death, similar WBC and platelet counts as compared to combined RT and RIT), the simultaneous administration of RT and RIT gave the longest tumor RD and the smallest MV ($P < 0.001$) at comparable toxicity levels.

Biodistribution Studies. Col12 tumor was selected for this experiment, because it gives relatively low but specific antibody uptake and presents quite well the average clinical situation. In the nonirradiated tumors, uptake of radiolabeled MAbs was rapid and reached 10.3% ID/g as early as 6 h after injection. Tumor radioactivity increased progressively until 24 h after injection, when it reached a mean maximum of 13% ID/g. Clearance of radiolabeled MAbs from the tumor was much slower than that from normal organs. As late as 7 days after injection, radioactivity in the tumor was still 11.2% ID/g. Nonspecific tumor localization of ^{125}I-labeled control IgG was measured in all mice. It was lower than the localization of specific MAbs, with a mean value of 7.3% ID/g at 24 h and 5.7% ID/g at 7 days after injection. These data are very similar to the earlier observations (24).

Table 4 *RDs, MVs, and skin toxicity after RT of different dose and fractionation schedules*

RT in Gy (fractionation)	No. of mice	RD[a] (days)	MV[b] (%)	Skin toxicity
20 (10 fractions, 12 days)	6	19.0 ± 9.1	100.0 ± 0.0[c]	0 of 6
30 (10 fractions, 12 days)	9	34.1 ± 7.1	82.7 ± 24.9	0 of 9
30 (10 fractions, 5 days)[d]	11	34.9 ± 6.7	35.2 ± 19.7	0 of 11
40 (10 fractions, 12 days)	10	39.3 ± 8.3	33.7 ± 23.6	0 of 10
50 (10 fractions, 12 days)	10	61.1 ± 5.6	13.3 ± 7.7	10 of 10[e]
50 (20 fractions, 12 days)[d]	5	41.3 ± 4.2	31.8 ± 7.3	4 of 5[f]

[a] Mean absolute tumor RD (±SD).
[b] Mean tumor MV (±SD).
[c] No tumor shrinkage was observed in these mice.
[d] Two fractions per day, 7–8 hours apart.
[e] 9 of 10 mice had skin toxicity grade III and 1 mouse had grade II.
[f] One of five mice had skin toxicity grade II, and three mice had grade I.
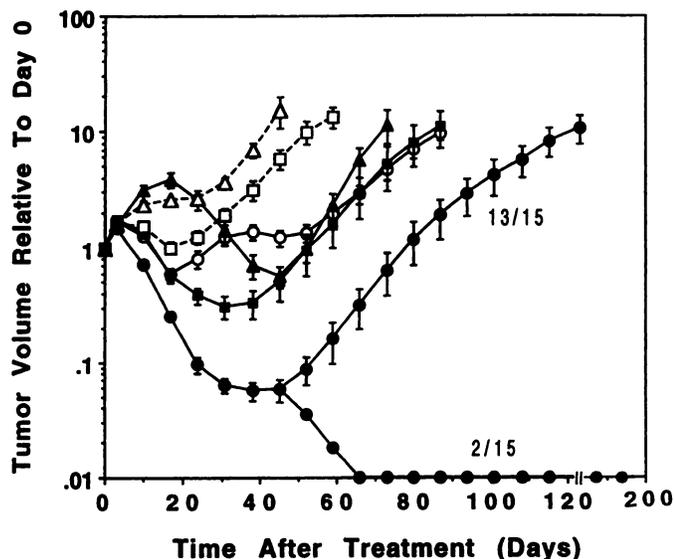
Fig. 3. Effect of 30 Gy RT, RIT, or the combined therapies on growth of Col12 xenografts in nude mice. The mean tumor volume relative to the initial tumor volume at day 0 is plotted *versus* time for each group (±SE). Definition of the groups is the same as in the legend to Fig. 2. △, RIT ($n = 7$); □, RT ($n = 9$); ○, RT-2W-RIT ($n = 12$); ▲, RIT-RT; ($n = 13$); ■, RT-RIT ($n = 12$); ●, RT+RIT ($n = 15$); bars, SE.

Table 5 *Blood analysis of mice 14 days after the last injection of ^{131}I-labeled MAbs in various combined therapy arms compared with RIT alone or untreated controls*

Treatment groups (No. of Mice)	Leukocytes No./mm³ (mean ± SD)	Platelets No./mm³ ($\times 10^5$; mean ± SD)
Untreated controls (24)	7406 ± 1280	13.8 ± 1.7
RIT alone (7)[a]	1913 ± 954	8.0 ± 1.9
RT+RIT (15)[b]	926 ± 728	7.0 ± 5.1
RT-RIT (12)	750 ± 430	5.9 ± 2.3
RIT-RT (13)	954 ± 553	8.4 ± 3.5
RT-2W-RIT (12)	1377 ± 1001	9.8 ± 3.5

[a] RIT, RIT of three injections of 200 μCi ^{131}I-labeled MAbs with a 1-week interval between injections.

[b] RT, RT of 30 Gy with 10 fractions over 12 days. Leukocyte and platelet counts were 1621 ± 744/mm³ and 11.7 ± 1.1 × 10⁵/mm³ at the end of RT and 5671 ± 974/mm³ and 11.6 ± 1.4 × 10⁵/mm³ 14 days after the end of RT, respectively ($n = 6$). Definitions of the combined therapies are the same as those in the legend to Fig. 2.

After irradiation with 6, 15 and 30 Gy, the tumor uptake of ^{131}I-labeled MAbs 24 h after injection was 14.1, 12.5, and 9.3% ID/g, respectively (Table 6). Thus, uptake of radiolabeled MAbs in tumors irradiated with 6 or 15 Gy was very similar to that of nonirradiated tumors, whereas the antibody uptake was decreased after 30 Gy irradiation ($P < 0.05$ as compared to nonirradiated tumors or 6 Gy-irradiated tumors).

Dosimetry. For one injection of 200 μCi of ^{131}I-labeled MAbs, the calculated tumor- and blood-absorbed radiation doses estimated from biodistribution studies were 18.0 Gy and 16.1 Gy, respectively (25). The radiation doses for normal tissues after repeated injections can be obtained by extrapolation from these data, whereas the dose for tumor tissue is not easy to extrapolate, because the % ID/g tumor could be lower after the second and third injections due to necrosis and partial saturation of the antigen by MAbs remaining from previous injections. However, the use of four MAbs for RIT with different epitope specificities makes the tumor saturation by antibody unlikely. Nevertheless, tumor dosimetry can only be indicative for these experiments.

Histology and Immunohistochemistry. Histology of fixed tissue sections of nonirradiated tumors showed that necrotic areas were observed only at a distance of 10 or more cell layers from supportive tissue (Fig. 5A). Occasional swollen cells and necrotic areas were

observed in tumors after RT of 6 Gy (Fig. 5B). RT of 30 Gy resulted in significant necrosis of tumor tissue (Fig. 5D), whereas an intermediate effect of some necrotic areas adjacent to supportive tissue was observed after 15 Gy irradiation (Fig. 5C).

Immunohistochemistry showed that CEA expressions by live tumor tissues were of similar intensity in untreated and irradiated tumors. Although 15 and 30 Gy RT produced significant tumor necrosis, CEA expression in the live tumor area was not reduced compared with that in untreated tumors (Fig. 5, E and F).

DISCUSSION

We have evaluated the effect of timing of an association of fractionated RIT and external-beam RT. The RT of 30 Gy to the tumor was given in a clinically relevant time schedule of five fractions per week. To counteract the radioresistance of a rapidly growing Col12 tumor, we used the fraction of 3 Gy, which is occasionally used clinically for palliative RT (30). The results indicate clearly that concurrent administration of RT and RIT gave the longest tumor RD and the smallest MV.

The simultaneous administration of both RT and RIT permits both modalities to act with the greatest intensity over the shortest period of

Time After Treatment (Days)
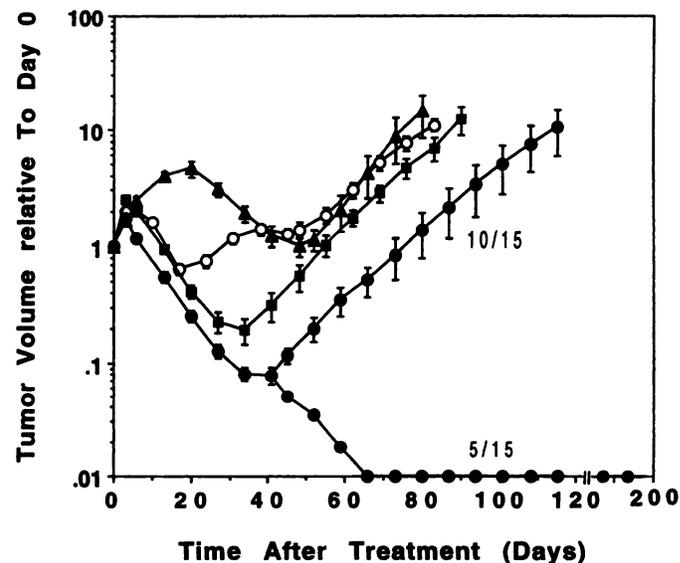
Fig. 4. Effect of combined 40 Gy RT and RIT on growth of Col12 xenografts in nude mice. The mean tumor volume relative to the initial tumor volume at day 0 is plotted *versus* time for each group (±SE). Definition of the groups is the same as in the legend to Fig. 2. In the RIT-RT group of eight mice, the one that had no tumor relapse is not included. ▲, RIT-RT ($n = 7$ of 8); ○, RT-2W-RIT ($n = 9$); ■, RT-RIT ($n = 11$); ●, RT+RIT ($n = 15$); bars, SE.

Table 6 *Biodistribution of the ^{131}I-labeled pool of four intact anti-CEA MAbs in mice bearing Col12 xenografts untreated or irradiated with 6, 15, or 30 Gy fractionated RT 24 h after MAb injection[a]*

The amount of injected MAbs was correlated with the RIT dose of 200 μCi per injection.

		Irradiation		
	Untreated	6 Gy	15 Gy	30 Gy
Tumor	13.0 ± 2.2	14.1 ± 2.8	12.5 ± 2.4	9.3 ± 2.3
Liver	5.5 ± 1.0	5.8 ± 1.0	5.3 ± 0.3	4.8 ± 1.3
Kidneys	3.3 ± 0.8	3.4 ± 0.5	3.1 ± 0.8	2.8 ± 0.8
Lungs	6.7 ± 1.5	6.7 ± 3.0	6.0 ± 2.2	7.5 ± 1.9
Spleen	3.9 ± 1.3	3.2 ± 1.4	3.7 ± 1.0	4.4 ± 1.4
Muscle	1.2 ± 0.3	1.5 ± 0.2	1.2 ± 0.1	1.1 ± 0.3
Blood	15.6 ± 2.7	16.1 ± 1.6	15.9 ± 1.6	14.5 ± 2.5

[a] Mean % ID/g (±SD) is shown.
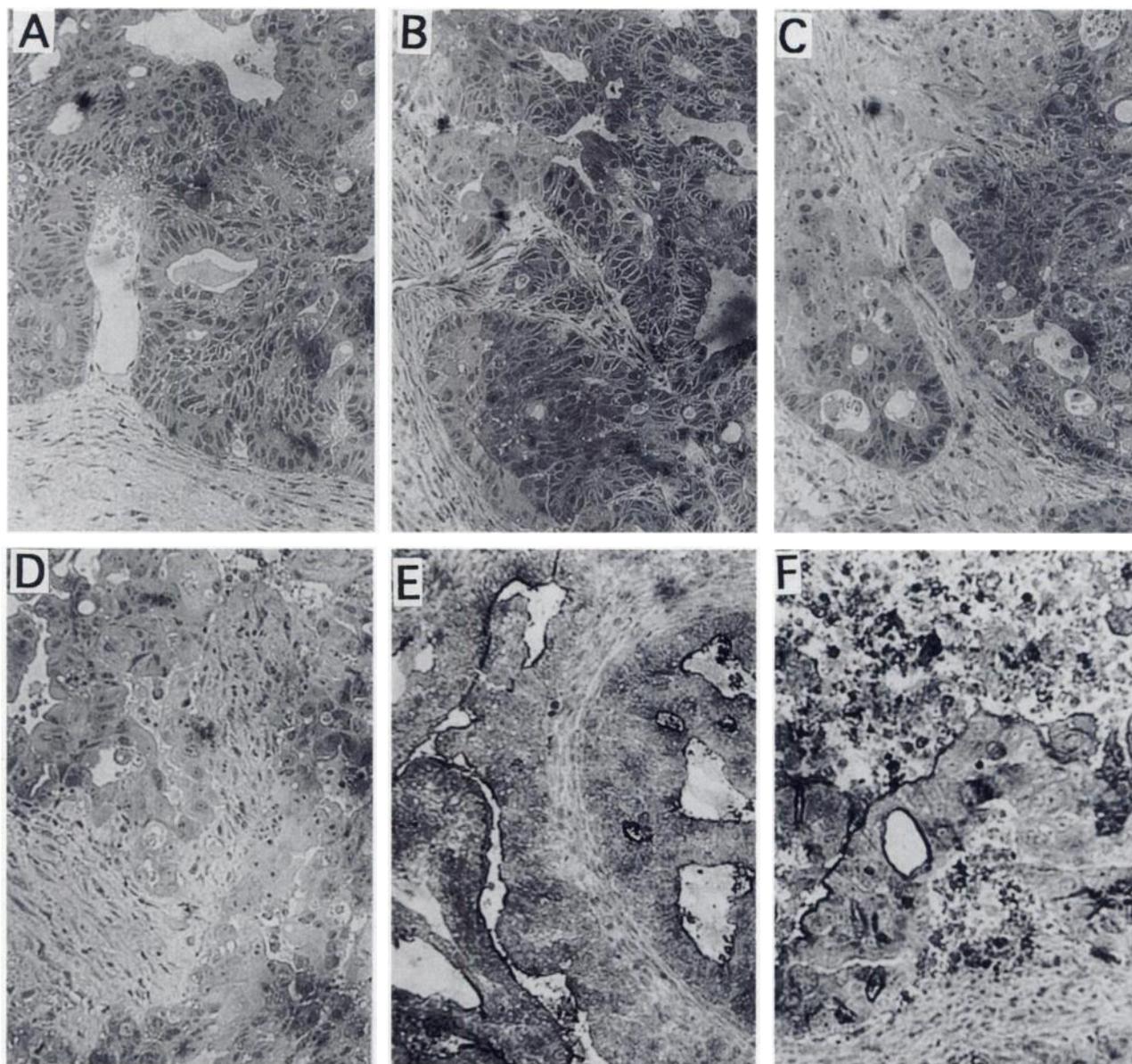
Fig. 5. Histology (A–D) and anti-CEA immunohistochemistry (E and F) of human carcinoma Co112 xenografts that were untreated (A and E) or irradiated with 6 Gy (B), 15 Gy (C) and 30 Gy (D and F). The tumor was irradiated with 3 Gy/fraction/day, (five fractions/week) and was removed for analysis 24 h after the last irradiation. In A and E, an intact epithelial tumor structure close to supportive tissue is visible, although some degree of tumor necrosis is present only at a distance from supportive tissue. B, occasional swollen tumor cells are detectable, but there is no significant necrosis. In C, and especially in D and F, a significant increase of necrotic tissue even adjacent to supportive tissue is demonstrated. E and F, anti-CEA immunohistochemical staining is shown in black. In E, the staining is restricted to pseudolumina, whereas in F, CEA-specific staining is more randomly scattered and is also presented in necrotic parts of the tumors, and the staining on live tumor tissue appears not to be reduced compared to untreated tumor.

time. Theoretically, even if RIT would have only a minimal cytotoxicity by itself, this effect might become significant when combined with an agent that has complementary cytotoxicity such as RT. According to the "multitarget" model of radiation damage, these synergistic effects might be due to the inhibition of repair of sublethal radiation damage or to the accumulation of ionizing hits in targets (31). Another potential speculative advantage of the simultaneous administration of RT and RIT could be that rapid killing of part of the tumor cells by RT could lead to a decrease of the interstitial pressure in the tumor. Furthermore, RT could produce a local inflammatory reaction, including an increase of vascular permeability. These two potential effects of RT could contribute to a better antibody distribution within the solid tumor nodule (32, 33) and increase the efficiency of RIT. Our study of radiolabeled antibody localization in irradiated tumors rather infirms this hypothesis, possibly because we used the fractionated RT instead of a single dose of irradiation. Indeed, we

have shown that antibody uptake in tumor after 6 and 15 Gy was similar to that of nonirradiated tumors. After a full irradiation dose of 30 Gy, however, the tumor necrosis and/or fibrosis reduced the antibody uptake to about 60% of the values observed in nonirradiated tumors. The overall results show that RIT can have an efficient antitumor effect when given concurrently with RT.

When analyzing the results of sequential RT and RIT as a function of the MV after treatment, distinct evolutions of tumor growth were observed during the three sequential treatment modalities. RT immediately followed by RIT resulted in the smallest MV among the sequential therapy schedules. The tumor volume was relatively small at the moment when RIT was started, and RIT contributed to further tumor shrinkage. In contrast, if RIT was used as a first therapy, tumor progression lasted longer due to the low-dose rate of radiation produced by RIT. RT in this group was therefore given at a moment when the tumor burden was quite large. When related to the initial tumor

size, regression was therefore less marked than that of RT followed by RIT.

RT followed after 2 weeks by RIT clearly demonstrates the disadvantage of introducing a delay between the two forms of radiation therapies. The intention in this timing was to hit the tumor cells with RIT at a time when the tumor volume was small due to the effect of RT and the BM had recovered from the radiation toxicity. The results show that tumor regrowth occurred even after initiation of RIT, the low dose rate of which was only able to limit the tumor proliferation for about 2 weeks. Thus, a separation of the two radiation modalities gave the tumor the opportunity to regrow and resulted in the biphasic growth curve.

In contrast, the sequential administration of RT and RIT produced similar tumor RDs independent of the order of treatment. We interpret this observation as indicating that overall, a similar percentage of cells were killed in the three sequential therapies. Interestingly, this is also the case in the groups in which RIT was given immediately or 2 weeks after RT.

When this experiment was repeated using an RT of 40 Gy instead of 30 Gy, the observations mentioned above were confirmed entirely. In addition, more local controls (5 of 15 mice) were observed in the simultaneous RIT combined with RT of 40 Gy, as compared to RT of 30 Gy (2 of 15). This encouraging result should provide a basis for additional experimental and clinical studies.

The toxicity of all combined RT and RIT regimens remained well within acceptable limits without significant weight loss, skin toxicity, and petechiae. The hematological toxicity did not change significantly among the various combined therapies. This good tolerance may be explained by the selective toxicity of each treatment alone. RIT does not increase radiation damage to the skin, and RT irradiates only part of the BM.

At the identical toxicity levels for skin (absence of skin lesions) or BM (similar WBC and platelet counts), the antitumor effects of simultaneous administration of RT (30 Gy) and RIT (three treatments of 200 μCi of ^{131}I-labeled MAbs) were superior to the maximum tolerated RIT alone (four treatments of 200 μCi of ^{131}I-labeled MAbs) or RT alone (40 Gy in 10 fractions over 12 days). Fifty-Gy RT produced skin toxicity despite being hyperfractionated into 20 fractions over 12 days.

In terms of isoeffective tumor doses for patients, it has been speculated that with a combination of RIT and RT, it might be possible to reduce external-beam RT by 10-20% while maintaining the tumor radiation dose constant (34). Alternatively, the combination of RIT with RT might allow an increase of the tumor radiation dose while maintaining toxic side effects within an acceptable limit that does not exceed those of either treatment alone. Additive therapeutic effects of combined, fractionated RT and RIT have been observed recently in nude mice with liver metastases of human colon cancer LS174T (35).

In view of the limited BM toxicity, absence of skin toxicity, and highly effective tumor regression and RD achieved by simultaneous administration of RT and RIT, we can conclude that this combined treatment has a therapeutic advantage as compared to the maximum tolerated RIT alone (where BM toxicity limits the dose) or to RT alone (where skin toxicity limits the dose). Furthermore, the significant increase of antitumor effect obtained by simultaneous administration of RT and RIT as compared to three sequentially combined schedules demonstrates the critical importance of timing in optimizing such therapy combinations. These findings may have clinical implications for designing the treatment protocols of colorectal cancer and other solid tumors.

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