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Research Article

"*Abscopal*" Effect of Radiation Therapy Combined with Immune-Therapy Using IFN-γ Gene Transfected Syngeneic Tumor Cells, in Rats with Bilateral Implanted N29 Tumors

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The tumor growth rate response was studied on N29 rat glioma tumor cells subcutaneously implanted on both hind legs of Fischer-344 rats. At around 30 days after inoculation, RT was given with ^{60}Co gamma radiation with 4 daily fractions of 5 Gy only to the right-lateral tumors. At days 26, 42, and 54 after inoculation, immunization was performed with irradiated syngeneic IFNγ-gene transfected cells. *Tumor growth rate* (TGR % per day) of the right-lateral irradiated tumor was significantly decreased ($P < 0.01$) after RT alone and with the combination of RT and immunization. But immunization alone gave no significant decrease of the TGR but significantly increased time of survival. The TGR of the unirradiated left-lateral tumors was significantly decreased ($P < 0.02$) only in the group of rats treated with RT alone. It is apparent that tumor cells killed by the radiation mediate suppression of tumor cells outside the target area. This effect is called the *abscopal* effect.

1. Introduction

Radiation therapy (RT) is usually focused on delivering the highest possible absorbed dose to the clinical target volume to destroy the tumor cells without exceeding the tolerance of surrounding normal tissue [1–3]. Effects on tumor cells outside the treated target area are mostly not considered in conventional radiation therapy regimes. In recent years, however, it has become increasingly apparent that radiation therapy can have an effect in tissues outside the immediate location of the radiation beam [4–7].

Nagasawa and Little (1992) observed that cells hit by α-particles, and neighboring cells without any hit, both exhibit the same type of damage. The phenomenon was called "bystander effect" borrowed from the field of gene therapy [8, 9]. Since then several reports and reviews have appeared dealing with this kind of nontarget effect also *in vivo* [10–19]. The bystander effect is, however, not often observed clinically [1, 16, 20].

Effects of radiation therapy on tumors outside of the radiation field have, however, been reported in many malignancies [1, 2, 21–30]. This phenomenon was originally described as abscopal effect by Mole in 1953 [31]. The definition of *abscopal* effect comes from the Latin ab (position away from) and scopus (mark or target).

The *abscopal* mechanism of action is still not fully explained, although it has been hypothesized that a variety of underlying biological events might contribute to the effect, including immune reactions and inflammatory response [32–34]. Immune-mediated abscopal effect has been observed in mice with 67NR tumor after RT with 2 or 6 Gy [5] and by studying the number of available dendritic cells (DCs), using the growth factor Flt3-Ligand(Flt3-L) [35]. Radiation therapy seems to augment the ability of

DCs to capture tumor antigens, for further homing to the draining lymph node, thereby mediating an effective antigen presentation that might play a vital role on the *abscopal* effect [36].

We have recently shown a strong enhancement of the therapeutic effect in intracranial N29 tumors by combining a single fraction of radiation therapy (5 as well as 15 Gy) and immunization with interferon-gamma (IFNγ) transfected immunogenic tumor cells [37, 38]. Previously we also presented a study demonstrating the abscopal effect of radiation therapy in a model of collaterally implanted N29 tumors in rats [6]. In the present study, we have used the same model of collaterally implanted tumors on both hind legs [6, 39], to investigate the non-target effect of radiation therapy combined with immunetherapy using IFNγ-gene transfected syngeneic tumor cells.

2. Materials and Methods

2.1. Animals. We used inbred female and male Fischer-344 rats weighing around 190 g and 370 g, respectively. The strain was maintained by continuous, single-line brother to sister mating in our laboratory. During the experiment, the rats were housed in a climate controlled cabinet. In the mean time, they were kept in Macralon cages provided with food pellets and water *ad libitum*. All experimental animal procedures were approved by the Animal Ethical Committee in Malmö/Lund (Lunds tingsrätt, Box 75, 22100 Lund Sweden).

The animals were observed daily for symptoms of the growing tumors, such as losing weight, unwillingness to move, shaggy fur, and reddening of the eyes and nose. When an animal developed such symptoms or the largest tumor exceeded 9 cm^3, it was euthanized.

The experiments A, B, C, and D were performed at different occasions over about one year. At the first experiment, A only 8 control rats (4 male and 4 female) were studied. The most extensive experiment was B, with 8 rats (5 male and 3 female) in each treatment group RT, IMU-IFNγ, and the combination IMU-IFNγ + RT. This experiment had two groups of controls with 8 rats in each: Gr 11 (5 male and 3 female) (see Figure 2(a)) and Gr 12 (4 male and 4 female). The 3rd experiment involved 8 control rats, and 9 rats were immunized. In the 4th experiment D, 7 rats (4 male 3 female) were used as controls, 7 (4 male and 3 female) rats were irradiated, and 2 rats were immunized. We aimed to have 8 rats (4 male and 4 female) in each group, but, due to the circumstances in breeding and competition with other experiments, the number and female/male ratio of rats in each group could vary. The number of controls and animals treated with radiotherapy (RT), immunization (IMU-IFNγ) or a combination (RT + IMU-IFNγ) of the two is summarized in the following Table 2. In total, 81 rats were involved in the entire study Table 1.

2.2. Cell Lines. The rat glioma N29 cell line was induced by administration of ethyl-N-nitro urea to 17-18-days pregnant Fischer rats. At 205 days, after administration, 80–90% of the offspring developed tumors in the central or peripheral nervous system. The cell line has been successfully propagated both *in vitro* and *in vivo*.

Table 1: Number of rats entered into each experimental series and groups of treatment.

Exp. series	Controls	RT	IMU-IFNγ	IMU-IFNγ + RT	All
A	8				8
B	16	8	8	8	40
C	8		9		17
D	7	7	2		16
All	39	15	19	8	81

Table 2: Time of immunization after inoculation (days) in the different groups of animals in series-B, -C, and -D.

Injection	IMU-IFNγ			IMU-IFNγ + RT
No.	Series B	Series C	Series D	Series B
1st	23	22	27	31
2nd	37	36	41	44, 51
3rd	51	50	55	61

All cells were cultured in antibiotic-free RPMI-1640 medium supplemented with 5–10% fetal calf serum, 2 mM L-glutamine, 10 mM HEPES, 0.5 mM pyruvate, and 0.096% $NaHCO_3$. Cell cultures were regularly checked for contaminating microbes by staining with the fluorescent dye Hoechst 32 258, examined with fluorescent microscopy. Cultures with suspected Mycoplasma infection were eliminated or treated with Mycoplasma Removal Agent (Hoechst, Germany) twice with 7 days interval and repeatedly confirmed free of infection. The cell cultures were maintained in culture flasks (Nunc, Denmark) and harvested by treatment with trypsin/EDTA.

2.3. Transfection of Cells for Immunization. Cells used for immunization were N29 tumor cells which were IFNγ gene transfected to enhance secretion of interferongamma. The IFNγ gene (The GenBank accession number for the genes is IFNγ, no. AF010466) was inserted into the cloning site (either the *Bam*HI or the *Eco*RI sites) of the retroviral vector pLXSN (GenBank accession no. M28248n). The gene constructs were sequenced, and 5 μg of plasmid DNA was subsequently used to transfect the retroviral packaging cell line GP1E86. Transfectam (Promega, Madison, Wis.) was used for transfect ion, according to the protocol of the manufacturer. The cell colonies which produced the highest number of retroviral particles were selected. Supernatants of these cells were used to infect the tumor cells. The infected tumor cells were cultured on selective media (Geneticin), and several single-cell clones were selected by limiting dilutions. The clones were checked for expression by either Northern blot analysis or semiquantitative polymerase chain reaction, and verified by studying the expression of the protein. After cell cloning, IFNγ production was evaluated by ELISA in supernatants that were harvested from tumor cells plated in 48-well plates, incubated for 48 hours at 37°C, and confirmed as 70 ng/10^6 cells. For immunizations, cells were

cultured for 1 week, washed twice, and suspended in medium [40].

2.4. Inoculation and Treatment of Subcutaneous Tumors. The rat glioma N29 was induced in our laboratory by subcutaneous administration in the hind legs. Two hundred thousand (200 000) cells were inoculated into the right leg whilst 50 000 cells were inoculated into the left leg in order to simulate a secondary smaller tumor. The tumor volume was estimated as an ellipsoid by length, width, and thickness measured with a caliper. When a tumor reached a volume of about 9 cm^3, was euthanized for ethical reasons. Tumors were treated about 4 weeks after inoculation when a solid tumor had developed with a diameter of 1–1.5 cm. Before treatment of the tumors, animals were anesthetized with 5% chloral hydrate given intraperitoneally (i.p.).

2.5. Immunization with IFNγ Gene Modified N29 Tumor Cells. The adenovirus transfected cells were transferred from the culture flasks with a cell density of 2×10^4 cells/mL in serum-free medium (IMDM-0) to 15 mL centrifuge test tubes (Nanclon) and stored in a melting ice bath before irradiation. The cells were radiation sterilized with an absorbed dose of 70 Gy in a Gammacell 2000 (Mølsgaard Medical, Risø, Denmark) at a dose rate of 4.0 Gy/min. During the irradiation, the cells were kept at room temperature. Directly after the irradiation they were placed in a melting ice bath. The sterilized cells are not proliferating but are secreting IFNγ for some time after administration to the rat.

The first immunization with sterilized cells was given five days before the radiation treatment and then two more times with 14-day intervals see Table 2. Immunization was performed with $3 \cdot 10^6$ IFN$_\gamma$ gene modified N29 tumor cells injected intraperitoneally.

2.6. Radiation Treatments. Radiation treatments were performed at around 30 days after inoculation. Before radiation therapy, the animals were anesthetized with 5% chloral hydrate given intraperitoneally (i.p.) or Ketalar/Rompun, 0.55 mL/100 g.

Animals were given fractionated radiation treatment using a ^{60}Co radiotherapy unit (Siemens Gammatron S) with a source-skin distance (SSD) of 50 cm and the maximum absorbed dose rate 0.65–0.70 Gy/min. A 0.5 cm thick, tissue-equivalent bolus (Super Flab, Mike Radio-nuclear instruments inc. NY, USA) was placed over the tumor to achieve full-dose buildup and a more homogeneous dose distribution in the tumor. The radiation field size was collimated to cover the tumor area with a margin of at least 1 cm (Figure 1).

The absorbed dose to the exposed right-lateral tumor was 5 Gy/day delivered at 4 consecutive days, that is, in total 20 Gy. The absorbed dose to the left-lateral tumor was less than 0.1 Gy. In previous experiments 20 Gy was shown to be suboptimal and noncurative dose suitable for studies of non-target effects [41]. The absorbed dose at various locations was measured both with an ionization chamber and TLD chip placed under the bolus.

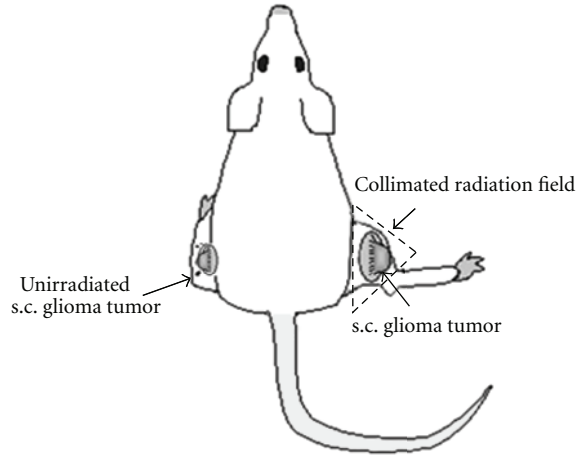


Figure 1: Experimental setup for radiation treatment.

2.7. Model for Tumor Growth Analysis and Synergistic Enhancement

2.7.1. Tumor Growth Rate (TGR). The tumor volume of each tumor was measured five days a week and fitted to a model of exponential growth. The tumor volume growth rate (TGR) of each individual tumor was estimated according to the following equations:

$$\frac{\partial \mathrm{TV}}{dt} = \mathrm{TGR} \cdot \mathrm{TV}, \qquad \mathrm{TV} = \mathrm{TV}_0 \cdot e^{\mathrm{TGR} \cdot (t - t_0)}, \qquad \ln[\mathrm{TV}] = \ln[\mathrm{TV}_0] + \mathrm{TGR} \cdot (t - t_0), \tag{1}$$

where TV is tumor volume at time t after inoculation, TGR is tumor growth rate constant day-1, TV0 is tumor volume at the time of radiation treatment $t = t_0$.

The TGR value was evaluated for each individual rat by a linear fit of ln(TV) at $t > t_0$.

2.7.2. Specific Therapeutic Effect (STE). The ratio of the tumor volume of the exposed tumor and corresponding control is a measure of the apparent surviving fraction, SF, of the cells in the treated tumor

$$\mathrm{SF} = \frac{\mathrm{TV}_{\mathrm{Exposed}}}{\mathrm{TV}_{\mathrm{Control}}}. \tag{2}$$

The therapeutic effect, TE, is a measure of the number of lethal events that has occurred in the cells of the treated tumor volume and thus defined as

$$\mathrm{TE} = -\ln(\mathrm{SF}) = \left[\mathrm{TGR}_{\mathrm{Control}} - \mathrm{TGR}_{\mathrm{Exposed}}\right] \cdot t. \tag{3}$$

In order to get a therapeutic effect measure independent of time, a quantity named "specific therapeutic effect" STE is defined. That is the tumor growth rate difference between the control and exposed tumor divided by tumor growth rate of the controls

$$\mathrm{STE}_i = \frac{\overline{\mathrm{TGR}^c} - \mathrm{TGR}_i^E}{\overline{\mathrm{TGR}^c}}, \tag{4}$$

where TGR_i^E is the individual tumor growth rate constant (day^{-1}) of *exposed* rats. $\overline{TGR}^c$ is the average of the individual tumor growth rate constant (day^{-1}) in the group of unexposed *control* rats.

The STE is equal to 0: when the average of tumor growth rate constant of the exposed group is equal to the average of the tumor growth rate constant of the control.

The STE is equal to 1: when the average tumor growth rate constant of the exposed group is equal to 0, which means arrested tumor growth.

The STE is larger than 1: when the average tumor growth rate constant of the exposed group is negative (<0), which means a declining tumor volume.

2.7.3. Specific Abscopal Effect (SAE). The "specific abscopal effect" SAE is defined as the tumor growth rate difference between the left-lateral unexposed tumor and corresponding control average divided by tumor growth rate of the average of left-lateral controls

$$SAE_i = \frac{\overline{TGR}^{UC} - TGR_i^{UE}}{\overline{TGR}^{UC}}, \quad (5)$$

where TGR_i^{UE} is the individual tumor growth rate constant of the unexposed (UE) left-lateral tumors in the group of N exposed rats. $\overline{TGR}^{UC}$ is the average of the individual tumor growth rate constant in the left-lateral tumors in the group of unexposed controls (UCs).

2.7.4. Therapeutic Effect Enhancement Ratio (TER). The enhancement effect the combined treatments is the ratio of the effect of the experimental combination (STE_{comb}) of the various treatment modalities and the therapeutic effect the hypothetically independent and additive combination of single treatment modality (STE_{ind}).

The therapeutic effect enhancement ratio of the exposed tumors is thus defined as:

$$TER = \frac{STE_{comb}}{\sum_i STE_{ind,i}}. \quad (6)$$

2.7.5. Abscopal Effect Enhancement Ratio (AER). The abscopal enhancement ratio of the left-lateral unexposed tumor is defined as

$$AER = \frac{SAE_{comb}}{\sum_i SAE_{ind,i}}. \quad (7)$$

The enhancement ratios TER and AER are measures of any synergistic or diminishing effect obtained in the combination of the various treatment modalities. It may be due to interaction of sublethal lesions induced by both treatment modalities to produce lethal events that cause the enhancement ratio >1. If the individual treatment modality is highly aggressive by itself, there might, however, also be an "over killing" effect that reduces the effect compared to the additive action, so that enhancement ratios <1. It is thus important to investigate the effect of combined treatments at various dose levels to find the maximum value of enhancement ratios.

2.8. Proliferation Assay. Nonadherent spleen cells (300 000/well) were plated onto 96-well flat-bottom plates and cultured with N29 glioma cells (15000/well) for 5 days. Coculture of nonadherent spleen cells with adherent spleen cells of rats from the various groups was performed by allowing 200 000 spleen cells (not previously subjected to plastic adherence) to adhere for 120 min in 96-well plates after which the non-adherent cells were removed.

Various numbers (50000, 150000, or 450000) of non-adherent spleen cells were added to these plates. The cells were cultured with Staphylococcal Enterotoxin A (SEA, 1 ng/mL) in the presence of N-nitro-L-arginine methyl ester (L-NAME, Sigma Chemical Co St Louis, Mo, USA).

Tritium-labeled thymidine (^{3}H-Thymidine) was added and kept during the final 6 h of culture. The cells were harvested on filter papers, scintillation fluid was added, and the radioactivity of the cells collected on the filter papers was measured in a beta counter (Wallac Microbeta, Turku, Finland). The recorded count rate in the tritium channel in counts per minute (cpm) was used as a measure of proliferation.

3. Results

3.1. Tumor Growth Rate TGR. The volume of each individual tumor was measured during the entire lifetime of all animals in each of the experimental groups. The Fischer-344 rats had N29 glioma tumors implanted on both thighs. But only the tumors on the right lateral side were irradiated.

The tumors are treated with radiation therapy (RT), immunization (IMU-IFNγ), and their combination (RT + IMU-IFNγ). The tumor volume was estimated by daily measurements five days a week. At those occasions the rats were also observed for symptoms from the tumor growth. In Figure 2, the tumor volume is displayed at each time of measurement of the animals of the series-B only. The tumor volume data fit well to an exponential growth model, and the fitted curve for all tumors in each group is displayed as a solid line in each diagram.

Tumor growth rate is estimated from the tumor volume measurements of each tumor fitted to a model of exponential growth,

$$TV = TV_0 \cdot e^{TGR \cdot (t - t_0)}, \quad (8)$$

where TV is tumor volume at time t after inoculation, TGR is tumor growth rate constant day^{-1}, TV_0 is tumor volume at the time of radiation treatment $t = t_0$.

The tumor growth-rate TGR was evaluated for each individual tumor starting at 30 days after inoculation when the radiation therapy took place. In Table 3 are given the averages of the results obtained from the growth rate of both left and right lateral tumors in the various series for controls,

and those treated with either RT, immunization (IFNγ), or their combinations are given.

The average tumor growth rate of the irradiated right-lateral tumor was 4.5 ± 0.3%/day which according to t-test is significantly decreased ($P < 0.001$) compared to the TGR of the controls 8.4 ± 0.3%/day. With immunization alone, however, the TGR 7.6 ± 0.6%/day was not significantly decreased compared to the controls. But, in a group given immunotherapy combined with RT, the TGR 5.7 ± 0.3%/day was significant decreased ($P < 0.001$) compared to the controls. In the group given RT to the right tumor, the TGR 6.1 ± 0.4%/day of the contra lateral unexposed left tumors is significantly ($P < 0.001$) reduced by 33% compared to the TGR 9.1 ± 0.3%/day of their controls. This effect on untreated tumors outside the target of irradiation is called the *abscopal* effect. In the group given RT to the right tumor combined with immunotherapy, the TGR 7.4 ± 1.0%/day of the contra lateral unexposed left tumors is decreased as well, however, not significantly compared to their controls.

Figure 3 displays the averages of the TGR results of all series. The results of the right treated tumors are displayed in blue piles and of the corresponding un-irradiated left-lateral bystander tumors are displayed in red.

3.2. Specific Therapeutic Effect and Specific Abscopal Effect. In order to estimate the therapeutic effect, we used the quantity "specific therapeutic effect" STE. The "specific therapeutic effect" STE is equivalent to the difference in tumor growth rate between the right controls and the right exposed tumors relative to the tumor growth rate of the right controls.

The corresponding quantity "specific *abscopal* effect" SAE was used for quantifying the *abscopal* effect. The "specific *abscopal* effect" SAE is equivalent to the difference in tumor growth rate between the average of the left controls and the left unirradiated tumors of the treated animals relative to the tumor growth rate of the left controls.

The STE and SAE values were calculated for each individual tumor. The values are normalized to the average of the controls of each experimental series and treated as one population. The averages of the results of each individual series are given in Table 4, and the pooled data of series-B, -C, and -D are displayed in Figure 4.

3.3. Therapeutic (TER) and Abscopal (AER) Enhancement Ratios. Therapeutic enhancement ratio (TER) and *abscopal* enhancement ratio (AER) at combined treatment with RT and IFNγ are derived as the ratios of the specific effects of the combined treatments and the sum of single treatments as given in Table 4. The value of the therapeutic enhancement ratio and the *abscopal* enhancement ratio of the combined treatments are

$$\frac{0.26}{0.46+0.09} = 0.47 \pm 0.07, \qquad \frac{0.14}{0.33-0.01} = 0.44 \pm 0.12, \tag{9}$$

respectively. Since these values are <1, there seems to be no synergistic effects of radiation therapy combined with immune therapy for extracranially implanted N29 glioma tumor.

3.3.1. Result of Proliferation Assay. The proliferation recorded in the spleen cell harvested from the various groups of rat is given in Table 5 as counts per minute recorded by the scintillation counter.

3.4. Result of Time to Sacrifice. When an animal showed symptoms of the growing tumors or the size of the tumor exceeded 9 cm^3, it was euthanized of ethical reason. The time of survival in the present study is thus time to sacrifice, which varied within each group and between the groups of various treatments. The result of time to sacrifice for the rats in experiment B where all types of treatments were involved is displayed in Table 6.

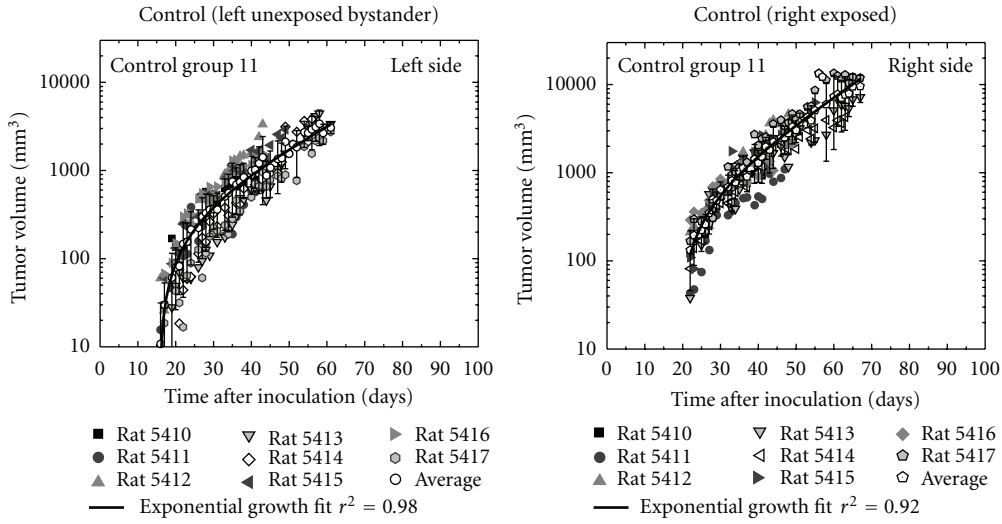
4. Discussion

4.1. Tumor Growth Rate (TGR). In the diagrams of Figure 2, it is shown that the tumor volume beyond 20–30 days after inoculation follows an exponential growth model. It is, however, not evident to draw quantitative conclusions out of the average growth curves because of the variation in the time of death between the rats. In order to perform a statistical analysis of the results, the tumor growth rate of each individual tumor is estimated from the daily tumor volume measurements of each tumor at 30 days after inoculation and thereafter. The averages of the results of the tumor growth rate thus obtained are summarized in Table 3, and displayed in Figure 3.

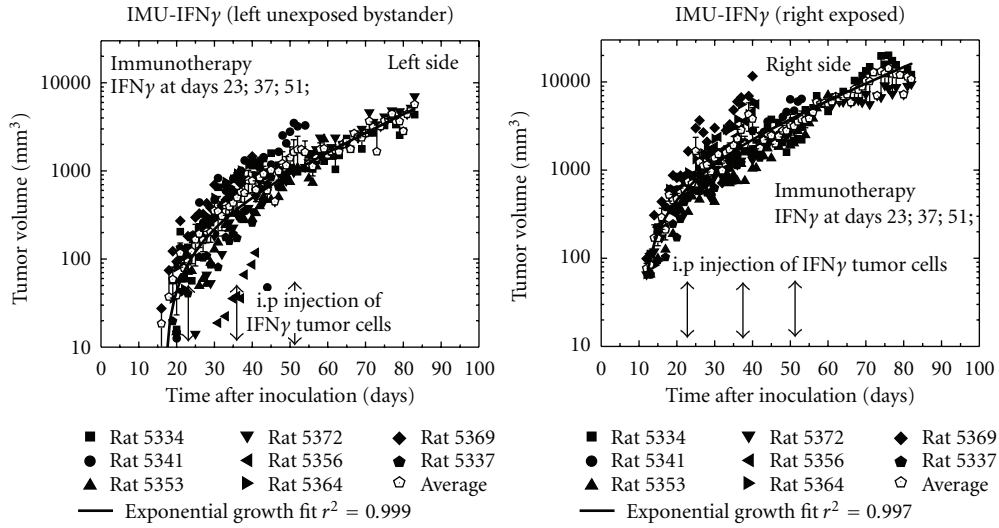
The tumor growth rate of the right-lateral tumor was significantly decreased for treatments with RT ($P < 0.0001$) compared to the controls. With immunization (IFNγ) alone in 19 rats, there was, however, no significant decrease of the TGR of the right lateral tumors compared to the controls. But in a group of 8 rats given the combination of immunization with RT, there was a highly significant decrease of the TGR values ($P < 0.001$) of the right irradiated tumors compared to the controls.

The TGR values are significantly reduced ($P < 0.0001$) on the contralateral unexposed left side in the group of 15 rats treated with RT only on the right tumor. This effect on untreated tumors outside the target of irradiation is called abscopal effect. With immunization (IFNγ) alone in 19 rats and with immunizations in combinations with RT in 8 rats, there were no significant decreases of the TGR in the left lateral tumors compared to the controls. Also the group of 8 rats given the combination of immunization and RT, there was no significant decrease of the TGR values of the left irradiated tumors compared to the controls. This is in agreement with previous findings that cellular immunization gives no antitumor response on tumors that produce immunosuppressive factors [42–44].

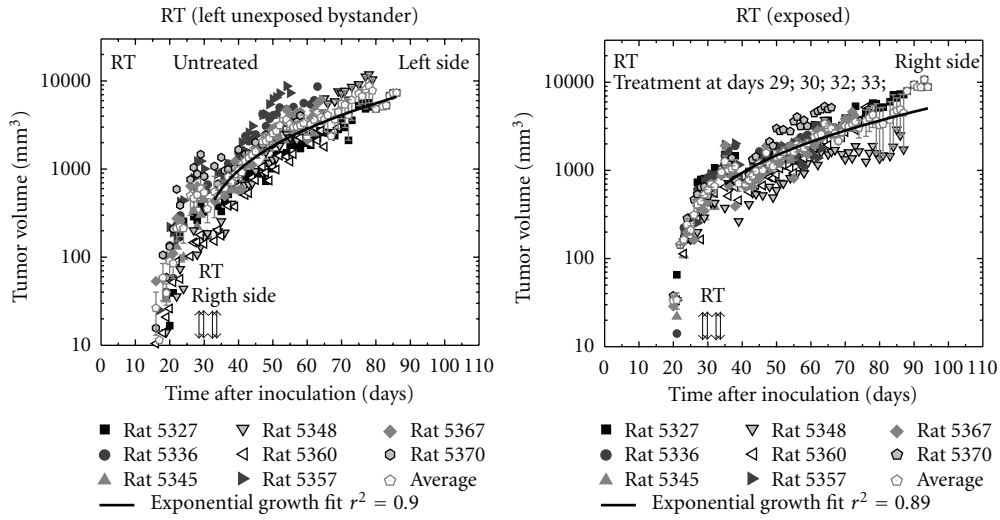
According to our previous results on single intracranial N29 tumors, radiation therapy was supposed to decrease the immune suppression by the tumor [37, 38]. But in the present model with two contralateral tumors the immune suppression by the untreated tumor seems suppress the action of the activated T cells.



(a) Tumor volumes at each time of measurement in all tumors of the controls in the series-B

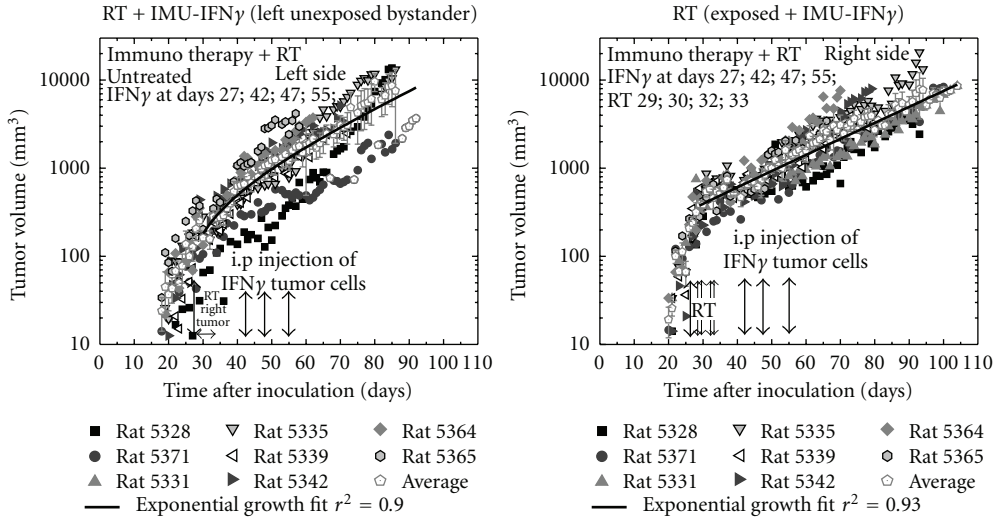


(b) Tumor volumes at each time of measurement in all tumors of the IMU-IFNγ treated rats in the series-B



(c) Tumor volumes at each time of measurement in all tumors of the RT group in the series-B

Figure 2: Continued.



(d) Tumor volumes at each time of measurement in all tumors of the RT+ IMU-IFNγ group in the series-B

Figure 2: The left diagrams show the tumor volume of the left-lateral untreated bystander tumors, and the right diagrams show the tumor volume of the right-lateral radiation-treated tumors. The solid lines show the curve fitted by an exponential growth model for $t > t_0$, that is, 30 days. In the left diagram of untreated tumors, the asterisk $(*)$ indicates the occasion of radiation treatment of the corresponding right-lateral tumors. In the right diagram for the right lateral treated tumors the thick arrow at "RT exposed" indicates the four radiation treatment fractions. The occasions of immunizations are indicated with double arrows.

Table 3: Tumor growth rate (% per day) of control rats and in rats after immunization with syngeneic IFNγ secreting cells (IFNγ). Right-lateral tumors were treated with radiation therapy (RT) while left lateral tumors were not treated. The P-values of t-test versus corresponding controls are given in the right columns.

Experimental series	Treatment	TGR left 50 k cells average ± SE	N	t-test versus Ctrl	Irradiated TGR right 200 k cells average ± SE	N	T-test versus ctrl
A	Controls	10.6 ± 0.6	8		9.4 ± 1.1	8	
B	Controls	8.7 ± 0.3	17		7.7 ± 0.3	17	
C	Controls	9.6 ± 0.6	8		9.1 ± 0.7	8	
D	Controls	7.8 ± 0.7	7		8.2 ± 0.8	7	
All	Controls	9.1 ± 0.3	40		8.4 ± 0.3	40	
A	IFNγ						
B	IFNγ	10.0 ± 1.9	8	ns	8.5 ± 1.3	8	ns
C	IFNγ	8.3 ± 0.4	9	ns	6.7 ± 0.6	9	0.02
D	IFNγ	9.9 ± 1.4	2	ns	8.3 ± 2.4	2	ns
All	IFNγ	9.2 ± 0.8	19	ns	7.6 ± 0.6	19	ns
A	RT						
B	RT	6.6 ± 0.6	8	0.01	4.4 ± 0.5	8	0.0001
C	RT						
D	RT	5.6 ± 0.3	7	0.02	4.5 ± 0.3	7	0.002
All	RT	6.1 ± 0.4	15	<0.001	4.5 ± 0.3	15	<0.001
A	IFNγ + RT						
B	IFNγ + RT	7.4 ± 1.0	8	ns	5.7 ± 0.5	8	0.006
C	IFNγ + RT						
D	IFNγ + RT						
All	IFNγ + RT	7.4 ± 1.0	8	ns	5.7 ± 0.5	8	<0.01

Table 4: Specific therapeutic effect (STE) and apecific *abscopal* effect (SAE) in each experimental series and groups of treatment after immunization 3 times with syngeneic IFN$_\gamma$ secreting cells.

Experimental series	Group of treatment	No RT SAE Left average ± SE	N	Left versus 0	RT : STE right average ± SE	N	Right versus 0
A	RT						
B	RT	0.24 ± 0.06	8	0.01	0.42 ± 0.06	8	<0.0005
C	RT						
D	RT	0.30 ± 0.04	7	0.001	0.49 ± 0.04	7	<0.0001
All	RT	0.33 ± 0.04	15	<0.0001	0.46 ± 0.04	15	<0.0001
1-9 A	IMU-IFNγ						
B	IMU-IFNγ	−0.17 ± 0.23	8	ns	−0.11 ± 0.16	8	ns
C	IMU-IFNγ	0.15 ± 0.05	9	0.02	0.26 ± 0.07	9	ns
D	IMU-IFNγ	−0.27± 0.18	1	ns	−0.02 ± 0.29	2	ns
All	IMUIFNγ	−0.01 ± 0.09	19	ns	0.09 ± 0.07	19	
1-9 A	IMU-IFNγ + RT						
B	IMU-IFNγ + RT	0.14 ± 0.12	8	0.01	0.26 ± 0.07	8	ns
C	IMU-IFNγ + RT						
D	IMU-IFNγ + RT						
All	IMU-IFNγ + RT	0.14 ± 0.12	8	0.001	0.26 ± 0.07	8	ns

Table 5: Proliferation measured as counts per minute, in spleen cells harvested from rats taken from the various groups of rats (No.: number of rats evaluated in each group).

No. added spleen cells	Control			IFNγ			RT			IFNγ + RT		
	Ave	SD	No.	Ave	SD	No.	Ave	SD	No.	Ave	SD	No.
450 000	35	±23	6	206	±172	5	37	±18	5	60	±79	4
150 000	16	±14	6	94	±54	5	27	±36	5	54	±74	4
50 000	65	±120	6	75	±49	5	6	±15	5	22	±16	4

4.2. Specific Therapeutic Effect and Specific Abscopal Effect. The "specific therapeutic effect" STE is obtained by normalizing the difference in tumor growth rate between the right-lateral controls and exposed tumors to the tumor growth rate of the right-lateral controls. The "specific *abscopal* effect" SAE is obtained by normalizing the difference in tumor growth rate between the left lateral controls and the unexposed tumor to the growth rate of the left lateral controls. The results of the *specific therapeutic effect* STE and the *specific abscopal effect* SAE are summarized in Table 4. These quantities are independent of time and normalized to the tumor growth characteristics of the controls of each experiment. In Figure 4, the averages of STE and SAE from each type of treatment are displayed.

4.2.1. Specific Therapeutic Effect (STE). For immunization alone with IFNγ secreting tumor cells, the average STE value became 0.09 ± 0.07, which is not significantly different from zero. The combination of RT with immunization resulted in a STE value of 0.26 ± 0.07 which is not significantly different from zero. But the STE value 0.26 for the combination is significantly lower ($P < 0.05$) than the therapeutic effect of RT for which STE value is 0.46 ± 0.04.

4.2.2. Specific Abscopal Effect (SAE). The specific *abscopal* effect SAE of the contralateral untreated tumors in rats treated with RT alone became 0.33 ± 0.04, which is significantly different from zero ($P < 0.001$). For immunization with IFNγ-transfected tumor cells, there was no effect on the contra lateral tumor. The SAE value in this case was −0.01 ± 0.09, which is not significantly different from zero. Immunization combined with RT resulted in a SAE value of 0.14 ± 0.12, which is neither significantly different from zero. Thus on the left tumor, there is no significant abscopal effect, of neither immunization alone nor immunization combined with RT.

4.3. Therapeutic (TER) and Abscopal Enhancement Ratio (AER). Our results do not show any enhanced non-target effects of radiation therapy combined with immunotherapy for extracranially implanted N29 glioma. But in another recent study with a similar model, the *abscopal* effect has been seen when RT is combined with immunotherapy [45]. In that study, it was found that fractioned (3 × 8 Gy) or (5 × 6 Gy) but not single-dose (20 Gy) RT induced an *abscopal* effect when the therapy was combined with anti-CTLA-4 antibodies in mice with colon and breast carcinoma cells. The reason why a single fraction of RT does not induce *Abscopal* effect might be the immunosuppressive function of immature myeloid cells [16, 46].

4.4. Mechanisms of the Abscopal Effect. The proliferation data given in Table 5 shows a significant enhancement

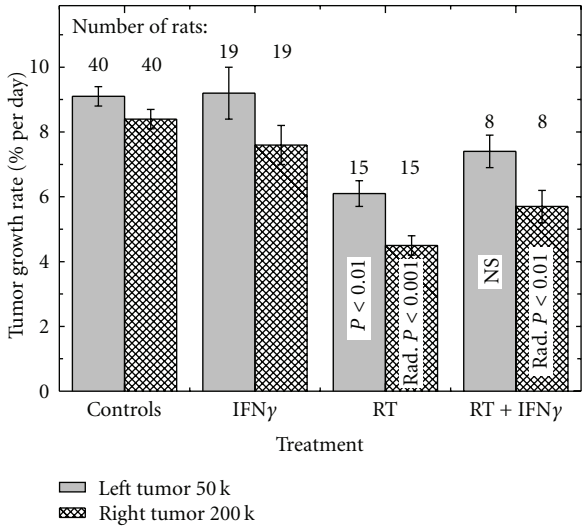


Figure 3: Tumor growth rate in rats with tumors in all series-A, -B, -C, and –D, with the average of the tumor growth rate for the four different treatment groups (controls, immunization (IMU-IFNγ), radiation therapy (RAD), or their combination (RAD + IMU-IFNγ) The averages of the TGR of the right lateral treated tumors is displayed in cross-pattern (right column), whereas the average of the tumor TGR of the corresponding untreated left-lateral tumors is displayed in gray (left column). The P-values (t-test) in the columns correspond to the TGR of the animals in the different treatment groups versus the TGR of the corresponding controls.

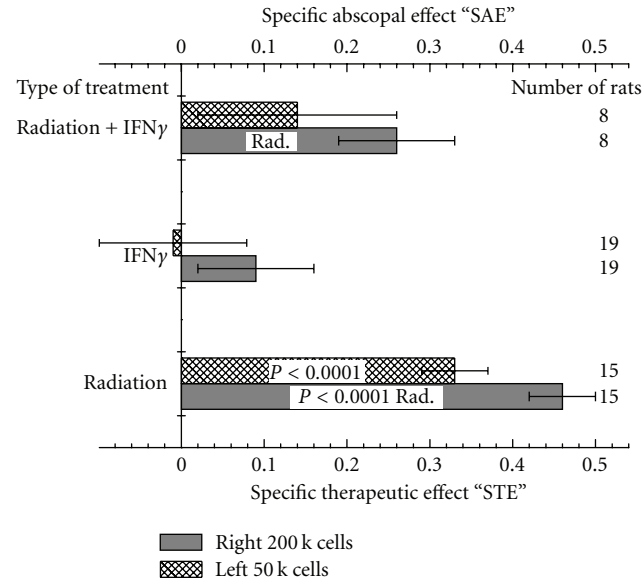


Figure 4: Specific therapeutic effect, STE (gray), and specific abscopal effect, SAE, (cross-pattern), from pooled data of series-B, -C, and -D. The p values (t-test) indicate the significance level of the STE and SAE being different from zero. No. is the number of rats in each group of various treatments.

when 150 k spleen cell from IFNγ cell immunized rat were analyzed. For the combination of RT and immunization with IFNγ gene transfected cell, there is slightly enhanced proliferation, although not significant. After RT only, no significant increase in the proliferation of spleen cells could be detected. The *abscopal* effect, however, is significantly increased after RT treatment as a single therapy which might indicate that the *abscopal* effect of RT might be mediated by radiation-produced specific factors.

It has been suggested by others that the *abscopal* effect might depend upon secretoric or clastogenic factors in plasma samples from RT patients [47]. It has also been observed that cells exposed with ^{60}Co γ-radiation produced a factor that mediates cell death in cells never exposed to radiation [11]. Other studies suggest that ionizing radiation induces the release of cytokines which mediate a systemic antitumor effect by activation of immune activity [33]. The existence of radiation-induced factors *in vivo* is now well accepted, and they are likely to be tissue and patient specific [10, 12]. Studies of the *abscopal* effect also focus upon pivotal roles of increased ceramide levels, leading to apoptotic signaling [14, 48]. The absorbed dose of 20 Gy applied in the present study is comparable to the absorbed dose of 15 Gy which has been used to raise ceramide levels in circulation that might induce apoptotic death of cancer cells also in nonirradiated areas [49]. Numerous reports have revealed that activation of secretoric acid sphingomyelinase (sSMase) by chemotherapeutic agents resulted raising intracellular ceramide levels and increased death of cancer cells [50, 51]. Generation of ceramide by radiation therapy provides a novel target for the elimination of tumors and opens a new avenue of treatment in those patients who have developed multidrug resistant cancer [52].

Immune cells such as macrophages and T cells which are activated through radiation exposure secrete cytokines including Il-1, Il-6, and TNFα, which lead to nitric-oxide-synthase (iNOS) activation and endogenous production of NO inside tumor cells. The nitric oxide NO diffuses out from the irradiated tumor and affects off-target tumor cells in a series of cascade reactions [34, 53]. It has been demonstrated that NO enhances the activation of the p53-dependent apoptotic program in tumor cells, which might be one of the mechanisms behind the *abscopal* effect [54]. Locally, enhancement of NO production has been used in a gene therapy approach by transfecting the iNOS gene into tumor cells combined with the radiation-inducible WAF1 promoter to drive the iNOS expression [55, 56].

Recent findings have also shown significant loss of global DNA methylation and a reduction of methyl-binding protein MeCP2 expression in both acutely radiation exposed and unexposed bystander spleen at 6 hr, 96 hr, and 14 days after irradiation [57]. This indicates that epigenetic effects might also be involved in the mechanism of the *abscopal* effect.

In a recent Japanese study with tumors implanted s.c. in both flanks of ECI301 mice, chemokine (human macrophage inflammatory protein-1 alpha variant) was injected i.v. after local irradiation (6 Gy) at the contra-lateral flank only. In about 50% of the treated mice, the nonirradiated tumor was completely inhibited. Leukocyte depletion studies suggest that CD8+ and CD4+ lymphocytes and NK1 cells were

Table 6: Time to sacrifice of rats in the various groups of experiment B.

Time of survival	Control			IFNγ			RT			IFNγ + RT		
	Ave	SD	No.	Ave	SD	No.	Ave	SD	No.	Ave	SD	No.
1-13	37	±8	16	56	±17	8	76	±10	8	86	±14	8
t-test versus control				$P = 0.02$			$P < 0.001$			$P < 0.001$		
t-test versus control							$P < 0.01$			$P < 0.005$		

involved. The results of that study indicate that chemokine administration after local irradiation might be useful for the treatment of advanced metastatic cancer [58]. It is thus apparent that tumor cells killed by the radiation contribute to establishing an immune response which is required for successful therapeutic effect and is partly protective. The immune response mediates the suppression of tumor cells outside the target area and affects the long-term survival of the patients. Recently, a functional relation was reported between the compounds HMGB1 released from the dying tumor cells activate the function of a toll like receptor (TLR4), which is important for the function of the immune system [59].

4.5. Time to Sacrifice. The time to sacrifice displayed in Table 6 indicates a significant increase of the time elapsed after inoculation until the rat was euthanized. For rat treated with immunization only the time to sacrifice increased about 50% compared to the untreated controls and for rats treated with radiotherapy only, the survival time increased about 112%. For the combined therapy (IMU-IFNγ + RT), the survival time increased about 131%. The average tumour size at time of sacrifice for the group of rats treated with IMU-IFNγ both alone and in combination with RT was about the same as the controls, while the tumor volume was reduced about 30% in the group of rats treated with radiation only. Thus, there seems to be a significant therapy effect of all treatments and in particularly RT and the combination (IMU-IFNγ + RT). But there was no significant difference in the time to sacrifice between RT only and the combined therapy (IMU-IFNγ + RT) which is in agreement with the TGR data. The time of sacrifice, however, gives no information about the *abscopal* effect which was the primary aim of the study.

5. Conclusion

By treatment of single intracranial N29 glioma tumors with RT in combination with immune therapy using IFNγ-transfected tumors cells, 75% complete tumor remissions have previously been demonstrated [37, 38]. That effect of radiation was related to diminishing the tumor's immune-suppression and enhanced the infiltration of activated T-cells affecting the tumor. In the present extracranial model with two contra-lateral tumors, it was hypothesized that activated T cells should also affect the left lateral unirradiated tumor. The results of RT alone appear as an *abscopal* effect with TGR decrease also in the contra-lateral un-irradiated tumor, which account for the *abscopal* effect. But in this model neither enhanced therapeutic nor *abscopal* effect was found by radiation therapy combined with immunotherapy using IFNγ-transfected tumor cells.

The mechanisms of RT-induced *abscopal* effect seems to be very complex involving several factors leading the activation of immune cells and apoptotic signaling [5, 14, 34, 45, 48, 53]. However, the *abscopal* effect opens for new and more effective radiation therapy regimes for spread disease. The combination of RT with increased ceramide levels, chemokine drugs, immune therapy, and gene therapy in order to enhance the *abscopal* effect should be further investigated.

Abbreviations

AER = $SAE_{Experimental}/SAE_{Independent}$:	*Abscopal* enhancement ratio
Ctrl:	Control
DC:	Dendritic cells
Gy:	Gray (J/kg)
IMU:	Immunization
IFNγ:	Interferon gamma
i.p.:	Intraperitoneally
RT:	Radiation therapy
N29:	Tumor cell line induced by administration of ethyl-N-nitro urea to pregnant Fischer rats
SAE = 1 − $TGR_{Un-Exposed}/TGR_{Ctrl}$:	SAE: specific abscopal effect
SSD:	Source-to-skin distance
STE = 1 − $TGR_{Exposed}/TGR_{Ctrl}$:	STE: specific therapeutic effect
TER = $STE_{Experimental}/STE_{Independent}$:	TER: therapeutic enhancement ratio
TGR:	Tumor growth rate % *per day*
TV:	Tumor volume
UC:	Unexposed controls
UE:	Unexposed tumors.

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