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**Research article** 

# Immunization with syngeneic interferon-gamma (IFN-γ) secreting tumour cells enhance the Therapeutic effect and Abscopal effect from combined treatment of subcutaneously implanted contra-lateral N29 tumours on Fischer rats with Pulsed electric fields (PEF) and <sup>60</sup>Co-gamma radiation.

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**Keywords:** Abscopal, Fischer rat, glioma, electric pulses, pulsed electric fields, PEF, radiation therapy, RT, immunization, syngeneic tumour cells, interferon-gamma, IFN- $\gamma$ .

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# Summary

The aim of the present study is to study the Abscopal regression of subcutaneously implanted N29 rat glioma after immunization with syngeneic IFN $\gamma$  secreting cells and treatment of contra-lateral tumours with pulsed electric fields (PEF) and/or radiation therapy (RT). The study was performed on rats of the Fischer-344 strain with rat glioma N29 tumours implanted subcutaneously on the flank or on both the right treated hind leg and the left untreated hind leg. Once weekly for three weeks, the animals were given intra-peritoneal injections of irradiated, modified N29 tumour cells, secreting interferon-gamma (IFN- $\gamma$ ). PEF was given with 16 exponentially decaying pulses at a maximum electric fields strength of 1400 V/cm and  $t_{1/e}$ = 1 ms. RT was given with <sup>60</sup>Co gamma radiation at daily fractions of 5 Gy, to a total absorbed dose of 20 Gy. The animals were arranged into controls and groups of various treatments: PEF, RT, PEF+RT and immunization (IFN $\gamma$ ).

Fitting the data obtained from consecutive measurements of tumour volume (*TV*) of each individual tumour to an exponential model  $TV = TV_0 \cdot exp[TGR \cdot t]$  estimated the *tumours growth* rate (*TGR %per day*) after the day of treatment (t = 0). TGR of the right-lateral treated tumour was significantly decreased for independent treatments with PEF and RT and with the combined treatment PEF+RT. With immunization (IFN $\gamma$ ) alone and in combination with PEF there was, however, no significant decrease of the TGR of the right-lateral tumours. But in the combination of immunization with RT or PEF+RT there was a highly significant decrease of the TGR values.

The Abscopal effect was evaluated by comparing the growth rate of the untreated contra lateral tumours with the treated tumours. TGR of the left-lateral untreated tumour in the groups with independent treatment of right-lateral tumours with PEF, was not significantly reduced. But the TGR values are significantly reduced in the group of rats treated with RT and the combination PEF + RT. With IFN $\gamma$  alone and in combinations with PEF or RT there was no significant decrease of the TGR in the left lateral tumours. But in the combination of IFN $\gamma$  with PEF+RT there was a highly significant decrease of the TGR values in the left lateral tumours.

The *specific therapeutic effect* (STE = 1 - TGR<sub>Exposed</sub>/ TGR<sub>Ctrl</sub>) after treatments with PEF was 0.30±0.01 and after RT 0.46±0.04 and after the combination PEF+RT 0.36±0.08. After immunization with IFN $\gamma$  secreting tumour cells the STE 0.09±0.07 is not significantly different from zero. Also for the combination of immunization and PEF the STE value of 0.07±0.07 is not significantly different from zero. In the combination of immunization with RT the STE value was 0.32±0.01 that is significantly different from zero and only slightly lower than for RT alone. The STE of the combination of immunization with (PEF+RT) resulted in an unexpectedly high STE value of 0.70±0.08 that is highly significantly different from zero (p < 0.0001).

The *specific Abscopal effect* (SAE = 1 - TGR<sub>Un-Exposed</sub>/ TGR<sub>Ctrl</sub>) of the contra lateral unexposed tumours in rats treated with PEF or RT are both significantly different from zero. For RT the average SAE value is  $0.33\pm0.04$  and for PEF it is  $0.11\pm0.05$ . The SAE value for the combined treatment with PEF + RT is  $0.26\pm0.02$  that is about the same as for RT alone. For immunization with IFN $\gamma$  secreting tumour cells only and IFN $\gamma$  +PEF the SAE values were not significantly different from zero. But IFN $\gamma$  combined with RT result in a SAE value of  $0.18\pm0.12$ 

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and the combination of IFN $\gamma$  with PEF+RT results in an improved abscopal effect with the SAE value of 0.33±0.06.

After combined treatment with PEF + RT the average of the therapeutic enhancement ratio  $(TER = STE_{Experimental} / STE_{Independent})$  is 0.47 ± 0.12 and the abscopal enhancement ratio ( $AER = SAE_{Experimental} / SAE_{Independent}$ ) is 0.61 ± 0.1 respectively. With all three treatment modalities combined IFN $\gamma$  + PEF + RT and all combinations of independent treatments with PEF, RT or IFN $\gamma$  are considered, the average of the TER is 1.20±0.15 and AER is 1.22±0.20. This might indicate that there is a synergism on the tumours on both sides by combining PEF, RT and immunization with IFN $\gamma$  secreting cells.

These results were first presented Nov 21-24, 2002, as Poster at Society of Neuro-Oncology (SNO) Annual Meeting, San Diego, USA (Persson et al 2002).

#### Abreviations

- $IFN\gamma = interferon gamma$
- PEF = pulsed electric fields
- RT = radiation therapy
- Gy = gray (J/kg)
- TV =tumour volume
- TGR = tumour growth rate
- $STE = specific therapeutic effect (STE = 1 TGR_{Exposed} / TGR_{Ctrl})$
- SAE = specific Abscopal effect (SAE = 1  $TGR_{Un-Exposed}/TGR_{Ctrl}$ )
- TER = therapeutic enhancement ratio ( $TER = STE_{Experimental} / STE_{Independent}$ )
- AER = abscopal enhancement ratio ( $AER = SAE_{Experimental} / SAE_{Independent}$ )
- GBM = glioblastoma multiforme
- GCG = giant cell glioma
- GFP = green fluorescent protein
- **DTH** = Delayed-Type Hypersensitivity

# I. Introduction

# A. Brain Immuno-Gene Tumour Therapy ("BRIGTT")

The most malignant type of brain tumours, glioblastoma-multiforme GBM or astrocytoma grade IV (according to the WHO classification) as well as giant cell glioma (GCG) is among the most therapy-resistant human cancers (Salford, et al., 1988). The tumour front of this tumour, consisting of highly proliferating cells, grows by progressively killing the surrounding normal brain cells, neuronal and glial. In autopsies, glioma cells, single or in small clusters, are demonstrated in the whole brain including the brain stem of patients with sub-cortical tumours (Burger et al., 1988). Radiotherapy and chemotherapy have hitherto proven not to be efficient enough for cure (Sheline 1977; Stenning, et al., 1987; Laperrieré et al., 2002; Berg et al 2003). But radiotherapy has been shown to be beneficial for patients of age under 50 year when treated for this type of tumour (Sandberg et.al 1991;Glinski et al., 1988).

We believe that the key for success in the treatment of glioblastoma multiforme would be a method to reach the migrating glioma cells, also called "guerrilla-cells". Such an efficient treatment regime could be "immunogenic tumour therapy". In a brain tumour rat model > 40% of the rats survived >30 weeks after immunization with syngeneic brain tumour cells transfected with the rat IFN- $\gamma$  gene. The cells were injected subcutaneously 1 to 3 days after the inoculation of non-transfected glioma cells in the brain of the rats. If not treated with immunisations, these animals develop lethal intra-cerebral tumours (glioma) within 3 to 4 weeks (Visse et al., 1999).

In a clinical research program *Brain Immuno-Gene Tumour Therapy* ("BRIGTT") approved by the Medical Products Agency in Sweden the immunisation take place by administration of radiation sterilised autologous tumour cells that have been genetically engineered to produce human interferon- $\gamma$  and green fluorescent protein (GFP) as immune enhancers, in the dermis of the upper arm (Salford et al., 2002, 2004; Baureus Koch et al. 2004). The method is safe for the patients and gives rise to positive DTH reactions and an increase of infiltrative CD8+ and CD4+ T cells at the site of immunisation. It is, however, not possible to conclude from the present patient material whether the method adds any beneficial effect. It may, though, be noted that two patients who received the full treatment with 10 immunisations survived for 27 months and another for 22 months after the pathological anatomical diagnosis. One patient has no MR signs of remnant tumour 15 months after diagnosis (Salford et al. 2004).

In order to further improve the therapeutic effect of immunotherapy we aimed to study the effect of combination with other therapeutic regimes. In the present work we investigate the effect of immunization with syngeneic IFN $\gamma$  secreting cells on Abscopal regression of contra-lateral implanted tumours in combination with pulsed electric fields and/or radiation therapy.

# B. Radiation therapy and the Abscopal effect

Radiation therapy is beside surgery still the most widely used therapy modality for cancer treatment and in developed countries it is given to one out of two patients with cancer (Knöös 1991, SBU 2003,Orth et al. 2014). Although a lot of progress is made in development of new methods and techniques for radiotherapy about half of the curatively intended treatments fail as a result of either distal or local recurrences (DeVita 1983). Improvements of local radiotherapy are focused on fractionation and higher absorbed dose to the clinical target volume without exceeding the tolerance of surrounding normal tissue (Suit et al. 1988, Suit and Miralbell 1989, Suit 2002). The therapeutic effect of radiation therapy refers to the direct exposed tumours and effects on tumours outside the treated target area are mostly not considered in radiation therapy.

Effects of radiation therapy on cancer tumours outside of the radiation field have, however, been reported in many malignancies (Nobler et al. 1969; Ehlers et al. 1973; Kingsley 1975; Antoniades et al. 1977: Rees et al. 1981; Rees and Ross 1983; Sham et al. 1995; Obha et al. 1998;Ludgate, 2012;Frey et al. 2012). This phenomenon was originally described as *abscopal effect* by R. J. Mole in 1953 (Mole 1953). The definition of *abscopal effect* comes from the Latin *ab* (position away from) and *scopus* (mark or target). The abscopal mechanism of action remains still unexplained, although a variety of under-lying biologic events can be hypothesized, including a possible role for the immune system (Uchida et al. 1989: Macklis et al. 1992). The Abscopal effect studied in mice with 67NR tumour after RT with 2 or 6 Gy, was recently proven to be an immune mediated effect (DeMaria 2004). The abscopal effect is, however, not often observed clinically, possibly because many tumour-bearing hosts develop immune suppression (Kusmartsev and Gabrilovich 2002).

Nagasawa and Little (1992) that exposed cells with  $\alpha$ -particles observed that cells hit by  $\alpha$ -particles and neighbouring cells without hit both exhibit same type of damage. The phenomenon was called "bystander effect" borrowed from the gene therapy field. Since then several reports and reviews have appeared dealing with this kind of nonlinear dose-response relationship that is called both bystander effect and abscopal effect (Mothersill and Seymour 2001, Azzam et al 2004). We have previously reported the Abscopal regression of subcutaneously implanted N29 rat glioma

after treatment of the contra-lateral tumors with pulsed electric fields (PEF) or radiation therapy (RT) and their combinations (PEF+RT) (Persson et al., 2004). Also the "Abscopal" Effect of Radiation Therapy Combined with Immune-Therapy Using IFN- $\gamma$  Gene Transfected Syngeneic Tumor Cells, in Rats with Bilateral Implanted N29 Tumors has recently been reported (Persson et al., 2011). The present report on the results of how Immunization with syngeneic interferon-gamma (IFN- $\gamma$ ) secreting tumour cells enhance both the Therapeutic effect and Abscopal effect from combined treatment of subcutaneously implanted contra-lateral N29 tumours on Fischer rats with Pulsed electric fields (PEF) and <sup>60</sup>Co-gamma radiation.

# C. The effect of pulsed electric fields

Pulsed Electric Field (PEF) treatment is the application of short, intense electric pulses used to increase the permeability of cell membranes and internalize exogenous molecules. Short electric

pulses of high field strength cause transient permeabilization of the cell membrane, allowing macromolecules, e.g. nucleic acids or genes, to access the cytoplasm without carrier. This has been employed *in vivo* to introduce anticancer agents into tumours (Engström et al. 1998, 2001a, Heller et al. 1995, 1996b,1997,1998; Jaroszeski et al. 1997a, 1997b, 1999, Salford et al. 1993), and recently also genes and proteins into tissues (Aihara 1998, Heller et al. 1996a, 2002, Mir et al. 1998, Nishi et al. 1996, Rols et al 1998). A series of clinical trials of this new cancer treatment modality has been performed and with encouraging results (Heller 1996b, 1999, Mir et al. 1998, Sersa et al. 1998).

Pulsed Electric Field (PEF) treatment can, however, by itself create cellular and sub cellular lesions by inducing lipid per-oxidation associated with the membrane area being permeabilized, and appears to be correlated to cell survival (Maccarrone et al. 1995a, 1995b). In biological peroxide can form hydroxyl radical OH that enable single strand breaks in DNA (Mello-Filho and Meneghini.1984). DNA damage from pulsed electric fields has thus been reported, where the number of DNA strand breaks was correlated to the field strength and duration of the electric pulses (Meaking, et al. 1994, 1995, Meldrum et al. 1999). Pulsed electric fields has also been found to induce apoptosis and activation of caspases (Hofmann et al. 1999, Pinero et al. 1997).

The tumour growth rate of the right-lateral treated tumours was significantly decreased for independent treatment with pulsed electric fields (PEF, p<0.005) but there was no significant decrease in tumour growth rate of the left-lateral tumour (Persson et al. 2004).

# D. Effect of Pulsed electric fields in combination with radiation therapy

The therapeutic effect of pulsed electric fields combined with radiation therapy was first studied in rats of the Fischer-344 strain with glioma tumours implanted subcutaneously on the thigh (Engström et al. 2001b). Exponentially decaying pulsed electric fields of 1400 V/cm and  $t_{1/e}=1$  ms were applied to the tumours with 16 pulses in the 4 consecutive days combined with radiation therapy of total absorbed radiation dose of 20 Gy, in 4 fractions of 5 Gy each day in four consecutive days. The combined treatment with pulsed electric fields was found to have a sensitizing effect on radiation therapy and resulted in a high fraction of complete remissions (6/9) and increased survival (60% at 100 days) (Engström et al. 2001b).

In a previous study in Fischer rats with a N29 tumour inoculated on both the right leg and on the left leg there is an enhanced therapeutic effect of the treated tumour as well as an Abscopal effect on distant non-treated tumours (Persson et al. 2004).

It would, however, be interesting to know if PEF and RT treatments affect the therapeutic effect of immuno-therapy. In the present study we use the same rat model with contra lateral subcutaneously implanted tumours to investigate the effect of immunization with transgenic tumours cells transfected with IFNy gene in combination with radiation therapy.

# **II MATERIALS AND METHODS**

# A. Experimental Animals and Tumour Inoculation

# 1. Animals.

Rats of the Fischer 344 strain were used. The strain was maintained by continuous, single line, brother/sister mating in our laboratory.

## 2. Tumour cell cultures.

All tumour cells were cultivated in antibiotic-free RPMI 1640 supplemented with 10% foetal calf serum, 2 mM L-glutamine, 10 mM Hepes, 0,5 mM pyruvate, and 0.096% NaHCO<sub>3</sub>. Cell cultures were maintained in culture flasks (Nunc, Denmark) and harvested with trypsin-EDTA.

## 3. Subcutaneous Tumours.

The rat glioma N29 was induced in our laboratory by subcutaneous administration in the hind legs. 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 tumour. Tumour volume is estimated as an ellipsoid by length, width and thickness measured with a calliper. When a tumour reached a volume of 9 cm<sup>3</sup>, the animal was sacrificed of ethical reasons.

# **B. Procedure of Tumour Treatment**

The effect of immunization with IFN $\gamma$  secreting cells, pulsed electric fields (PEF), radiation therapy (RT) and their combination was investigated in male rats of the Fischer-344 strain with rat glioma N29 tumours implanted subcutaneously on the flank or on the thigh of the hind leg. The animals were arranged into eight groups as shown in **Table 1**.

Group	Treatment	Number of rats
Ctrl	Controls with no treatment	40
RT	Radiation therapy 4×5 Gy	25
PEF	Pulsed Electric Fields	15
PEF+RT	Pulsed Electric Fields + Radiation Therapy	15
IMU	Immunization	19
RT+IMU	Immunization + RT + Immunization	24
PEF+IMU	Immunization + PEF + Immunization	8

Table 1. Number of animals in each group of various treatments.

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PEF+RT+IMU	Immunization + PEF + RT + Immunization	14
Total		160

Tumours were treated about 4 weeks after inoculation when a solid tumour has developed with a diameter of 1-1.5 cm. Before treatment of the tumours, animals were anesthetized with 5% chloral hydrate given intra-peritoneal (i.p.).

# 1. Immunization with IFN-- $\gamma$ gene modified N29 tumour cells.

The adenovirus transfected cells were transferred from the culture flasks with a cell density of  $2\times10^4$  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 IFN- $\gamma$  for some time after administered to the rat.

The first immunisation with sterilized cells was given five days before the treatment and then two more times with 14-day intervals. Immunization was performed with  $3.10^6$  cells tumour cells injected intra-peritoneally.

#### Table 2

Time of m	infumzation arte	i moculation (days	s) in the different gr	oups of annuals in series-i	5, <b>-</b> C and <b>-</b> D.
THIC OF III	infumzation arte	i moeulation (days	<i>y</i> in the unificient <u>s</u> i	oups of annuals in series i	$\mathbf{D}$ , $\mathbf{C}$ and $\mathbf{D}$ .
Time of in	munization afte	r inoculation (days	s) in the different or	ouns of animals in series-l	3 -C and -D

Injection		IFNγ		$IFN\gamma + PEF$			IF	$FN\gamma + RT$	-	$IFN\gamma + (RT+PEF)$		
No.	serie	serie	serie	serie	serie	serie	serie	serie	serie	serie	serie	serie
	В	С	D	В	С	D	В	С	D	В	С	D
1st	23	22	27	21	22	26	31			41		26
2nd	37	36	41	36;42	36	41	44,51			56;64		41
3rd	51	50	55	50	50	55	61			70		55
4th												65

## 2. Pulsed Electric Field (PEF) treatment.

Two rectangular flat electrodes were mounted on a slide calliper and connected to an exponential pulse generator (Fig 1). In the first series of experiment we used a BTX600 (Genetronics, San Diego, USA) and since year 2000 a CythorExp 2000 (Aditus Medical AB, Lund Sweden) was used. The pulsed delivered was monitored by an oscilloscope and the load was adjusted so that the time constant of the exponential pulse was 1 ms. The hair over the tumour was shaved off and the skin was carefully covered with electro-cardiac paste to ensure good electrical contact between electrodes and skin. The paste also moistened the skin, reducing the transdermal impedance and limited the risk of skin necrosis from the pulse treatment. The tumours were gently fixed in position between the two electrodes, and the voltage was adjusted to the distance between the electrodes to deliver pulses of identical field strength to all tumours. Sixteen pulses of approximately 1400 V/cm with a time constant of 1 ms were delivered trans-dermally to the whole tumour during approx. 20 seconds. This treatment was repeated daily for four days. The delay

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between electro pulsation and radiation treatment was kept as short as possible (4-5 minutes).



Figure 1. Experimental set-up for radiation treatment and electric impulse treatment

#### 3. Radiation treatment (RT).

Animals were given fractionated radiation treatment using a <sup>60</sup>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 tumour to achieve full dose build-up and a more homogeneous dose distribution in the tumour. The radiation field size was collimated to cover the tumour area with a margin of at least 1 cm (**Fig 1**). The absorbed dose to the exposed right-lateral tumour was 5 Gy/day  $\times$  4 (consecutive) days, in all 20 Gy, which in preparatory experiments showed to be a suitable, suboptimal and non-curative dose. While the absorbed dose to the left-lateral tumour was negligible.

# C. Model for Tumour Growth Analysis and Synergistic enhancement

The tumours are treated with Pulsed Electric Fields (PEF) Radiation therapy (RT), a combination of these two (PEF+RT) and in combination with immunotherapy. The question is how to quantitatively express the enhancement of the therapeutic effect of the experimentally combined treatments with hypothetical independent combinations of the single treatments.

#### 1. Tumour growth rate

The tumour volume measurements of each tumour fitted to a model of exponential growth made the tumour volume growth rate (TGR) according to the following equation.

$$\frac{\partial TV}{dt} = TGR \cdot TV; \qquad TV = TV_0 \cdot e^{TGR \cdot t}$$

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where		

TV	Tumour volume
TGR	Tumour growth rate constant day <sup>-1</sup>
$TV_0$	Tumour volume at the time of treatment

The therapeutic effect is defined as the ratio of the tumour volume between the treated tumour and the control group.

## 2. Specific Therapeutic Effect (STE)

The ratio of the tumour volume of the exposed tumour and corresponding control is a measure of surviving fraction, *SF*, of the cells in the treated tumour:

$$SF = TV_{Exposed} / TV_{Control}$$

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

$$TE = -ln(SF) = [TGR_{Control} - TGR_{Exposed}] \cdot t$$

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

$$\text{STE} = \frac{1}{N} \sum_{i=1}^{N} \frac{\overline{TGR}^{C} - TGR_{i}^{E}}{\overline{TGR}^{C}};$$

where

 $TGR_i^E$  The average of the individual Tumour growth rate constant (day <sup>1</sup>) in the group of N **exposed** rats.

 $\overline{TGR}^{C}$  The average of the individual Tumour growth rate constant (day<sup>-1</sup>) in the group of unexposed **control** rats.

The STE is equal to 0 when the average of tumour growth rate constant of the exposed group, is equal to the average of the tumour growth rate constant of the control

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

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The STE is larger than 1 when the average tumour growth rate constant of the exposed group, is less than 0, which means a declining tumour volume.

#### 3. Specific Abscopal Effect (SAE)

The "specific abscopal effect" SAE is defined as the tumour growth rate difference between the left-lateral unexposed tumour and corresponding control divided by tumour growth rate of the controls.

$$SAE = \frac{1}{N} \sum_{i=1}^{N} \frac{\overline{TGR}^{UC} - TGR_i^{UE}}{\overline{TGR}^{UC}};$$

where

 $TGR_i^{UE}$  The average of the individual tumour growth rate constant of the unexposed (UE) left-lateral tumours in the group of N exposed rats.

 $\overline{TGR}^{UC}$  The average of the individual tumour growth rate constant in the left-lateral tumours in the group of unexposed controls (UC).

#### 4. The therapeutic enhancement ratio and Abscopal effect enhancement ratio

The enhancement effect the combined treatments is the ratio of the effect of the experimental combination of the various treatment modalities and the therapeutic effect the hypothetical independent combination of single agents.

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

$$TER = \frac{STE_{Experimental}}{STE_{Independent}}$$

and

the abscopal enhancement ratio of the left-lateral unexposed tumour is defined as:

$$AER = \frac{SAE_{Experimental}}{SAE_{Independent}}$$

where the hypothetical effect (..E) by independent (additive) action of ionizing radiation (RT), pulsed electric fields (PEF) and immunization (IFN $\gamma$ ) is given by

$$..E_{Independent} = ..E_{RT} + ..E_{PEF} + ..E_{IFN\gamma}$$
, or

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	$E_{Independent} =E_{RT+PEF} +E_{IFN\gamma}$ , or	
	$E_{Independent} =E_{PEF+IFN\gamma} +E_{RT}$ or	
	$E_{Independent} =E_{RT+IFN\gamma} +E_{PEF}$ , or	

The enhancement ratios are measures of any synergistic or diminishing effect obtained in the combination of the various agents. It may be due to interaction of sub lethal lesions induced by both agents to produce lethal events that cause the enhancement ratio > 1. If the individual therapies are highly aggressive by themselves there might, however, also be an "over killing" effect that reduce 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.

# **III. Results**

The volume of each individual tumour was measured during the entire lifetime of all animals in the following experimental groups. The female Fischer-344 rats had N29 glioma tumours implanted on both thighs and only the tumours on the right lateral side were treated.

Exp.				PEF+R					
Series	Controls	PEF	RT	Т	IFNγ	IFNγ	IFNγ	IFNγ	
						+ PEF	+ RT	+PEF+RT	All
А	8								8
В	17	9	8	7	8	8	8	7	72
С	8	9			9	9			35
D	7	7	7	8	2	7		7	45
All	40	25	15	15	19	24	8	14	160

Table 3. Number of animals entered into each experimental series and groups of treatment.

The tumours are treated with Pulsed Electric Fields (PEF), radiation therapy (RT), and Immunization (IFN $\gamma$ ) and all possible combinations (PEF +RT) (PEF+IFN $\gamma$ ) (RT+IFN $\gamma$ ) (PEF+RT+IFN $\gamma$ ).

In the series-B the right-lateral tumours were treated with four fractions of exponential Pulsed Electric Fields (PEF) radiation therapy (RT) and their combination (PEF+RT). The PEF treatment was performed at days 42, 43, 45 and 46 after inoculation of the tumours by applying 16 exponential pulses at electric field strength of 1400 V/cm, and 1.0 ms time constant with plate electrodes over the tumours. The radiation therapy was performed at days 29, 30, 32 and 33 after inoculation with four daily fractions of 5 Gy (total 20 Gy). The combined treatment was performed with less than 1 h between the PEF and RT treatments performed as above at days 30,

#### 31, 33 and 34 after the day of inoculation.

The tumour volume was estimated by more or less daily measurements. At those occasions the rats were also observed for symptoms from the tumour growth. The Figure 2 displays the average tumour volume at each time of measurement of tumours in the animals of the series-B. At about 30 days after inoculation and thereafter the tumour growth data fit well to an exponential growth model. The fitted curves for all tumours in each group are displayed as solid lines.

The effect of immunization was studied by intra-peritoneal injection of IFN $\gamma$  secreting syngeneic tumour cells at three occasions in about 10-20 days interval during the experiment. The first immunisation with sterilized cells was given five days before the treatment and then two more times with 14-day intervals. Immunization was performed with  $3 \cdot 10^6$  cells tumour cells injected intraperitoneally. The time of immunization after inoculation (days) in the different groups of animals in series-B, -C and –D are given in **Table 2.** The diagrams in **Figure 2b** display the average tumour volume at different time of measurements of rats of the experimental series-B immunized by intra-peritoneal injection of IFN $\gamma$  secreting syngeneic tumour cells alone or in combination with PEF, RT or PEF + RT. The p-values of significant difference of the TGR versus corresponding controls and right treated tumours versus left untreated tumours are given in the right columns of **Table 4**.

Tumour growth rate is estimated from the tumour volume measurements of each tumour fitted to a model of exponential growth,  $TV = TV_0 \cdot exp[TGR \cdot t]$ . Where "TV" is Tumour volume, "TGR" is tumour growth rate constant (day<sup>-1</sup>) and t is time after first treatment. The results thus obtained from the growth rate of right lateral tumours in all series-A, -B, -C, and -D with average of treated with either PEF, RT, (PEF+RT) and immunization (IFN $\gamma$ ) or their combinations are given in **Table 4.** In **Figure 3** the averages of the TGR results of all series of the right treated tumours are displayed in red columns and of the corresponding untreated left-lateral tumours are displayed in green.



#### Figure 2a

Average tumour volume at each time of measurement of tumours in the animals of the series-

B. The left stack of diagrams show the tumour volume of the left-lateral untreated tumours and the right stack of diagrams shows the tumour volume of the right-lateral treated tumours. The solid lines show the fitted exponential growth model with the growth rate constant "TGR" given in the lower right corner of each diagram. In the left diagram of untreated tumours the \* indicate the occasion of treatment of the corresponding right lateral tumours. In the right diagram for the right lateral treated tumours the arrows indicate the 4 treatment fractions.



#### Figure 2b

Average tumour volume at each time of measurement of tumours in the animals of the series-B with immunization by syngeneic IFN $\gamma$  secreting tumour cells. The occasions of i.p. injection of the IFN $\gamma$  secreting tumour cells are marked with 'I' in the diagrams. The left stack of diagrams show the tumour volume of the left-lateral untreated tumours and the right stack of diagrams shows the tumour volume of the right-lateral treated tumours. The solid black lines show the fitted exponential growth model with the growth rate constant "TGR" given in the lower right corner of each diagram. In the left diagram of untreated tumours the \* indicate the occasion of treatment of the corresponding right lateral tumours. In the right diagram for the right lateral treated tumours the arrows indicate the 4 treatment fractions.

#### Table 4 a

Experiment 1 Series	a Treatment	Average SE Right	N	Average SE Left	N	t –test vs. ctrl Right	t-test vs. Ctrl Left	t –test Right vs. Left
А	Controls	9.4 ±1.1	8	10.6 ±0.6	8	1.0	1.0	0.14
В	Controls	7.7 ±0.3	17	8.7 ±0.3	17	1.0	1.0	0.03
С	Controls	9.1 ±0.7	8	9.6 ±0.6	8	1.0	1.0	0.19
D	Controls	8.2 ±0.8	7	7.8 ±0.7	7	1.0	1.0	0.61
All	Controls	8.4 ±0.3	40	9.1 ±0.3	40	1.0	1.0	0.02
A B	PEF PEF	5.1 ±0.9	9	7.2 ±0.7	9	0.03	0.08	< 0.0001

Tumour Growth Rate (% per day) of right lateral tumours treated with PEF, RT and PEF+RT and of left lateral untreated tumours. The p-values of t-test versus controls and treated versus untreated tumours are given in the right columns.

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С	PEF	6.7 ±1.4	9	9.9 ±0.6	9	0.17	0.74	
D	PEF	$5.7 \pm 0.6$	7	7.1 ±0.6	7	0.03	0.47	< 0.0001
All	PEF	5.9 ±0.6	25	8.1 ±0.4	25	0.003	0.287	0.002

continued

Experimenta	a							
1	Treatment	Average SE	Ν	Average SE	Ν	t –test vs.	t-test vs.	t –test
Series		Right		Left		ctrl Right	Ctrl Left	Right vs. Left
А	RT							
В	RT	4.4 ±0.5	8	$6.6 \pm 0.6$	8	0.0001	0.01	0.06
С	RT							
D	RT	4.5 ±0.3	7	5.6 ±0.3	7	0.002	0.02	0.04
All	RT	4.5 ±0.3	15	6.1 ±0.4	15	0.00000003	0.000033	0.01
А	PEF+RT							
В	PEF+RT	6.0 ±1.3	7	6.7 ±0.3	7	0.26	0.0004	0.58
С	PEF+RT							
D	PEF+RT	$4.9 \pm 0.4$	8	6.7 ±0.2	8	0.004	0.16	0.01
All	PEF+RT	5.4 ±0.7	15	6.7 ±0.2	15	0.003	0.00003	0.06

#### Table 4 b

Tumour Growth Rate (% per day) after Immunization with syngeneic IFN $\gamma$  secreting cells. Right lateral tumours treated with PEF, RT and PEF+RT and of left lateral untreated tumours. The p-values of t-test versus controls and treated versus untreated tumours are given in the right columns.

Experimental Series	Treatment	Average SE Right	N	Average SE Left	N	t –test vs. ctrl Right	t-test vs. Ctrl Left	t –test Right vs. Left
А	IFNγ							
В	IFNγ	8.5 ±1.3	8	$10.0 \pm 1.86$	8	0.54	0.48	0.27
С	IFNγ	6.7 ±0.6	9	8.3 ±0.4	9	0.02	0.08	0.05
D	IFNγ	8.3 ±2.4	2	9.9 ±1.4	2	0.79	0.68	0.76
All	IFNγ	7.6 ±0.6	19	9.2 ±0.8	19	0.28	0.93	0.03
А	IFNy + PEF							
В	IFNγ + PEF	8.9 ±0.4	8	11.2 ±1.3	8	0.02	0.11	0.12
С	$IFN\gamma + PEF$	7.1 ±1.5	9	10.8 ±1.3	9	0.27	0.40	0.01
D	$IFN\gamma + PEF$	7.5 ±0.6	7	$8.8 \pm 0.5$	7	0.45	0.26	0.18
All	IFNy + PEF	7.8 ±0.6	24	10.3 ±0.7	24	0.41	0.09	0.00
А	$IFN\gamma + RT$							
В	$IFN\gamma + RT$	5.7 ±0.5	8	7.4 ±1.0	8	0.006	0.29	0.0002
С	$IFN\gamma + RT$							
D	$IFN\gamma + RT$							
All	$IFN\gamma + RT$	5.7 ±0.5	8	7.4 ±1.0	8	0.001	0.17	0.00003

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	А	IFNy PEF+RT							
	В	IFNy PEF+RT	$1.5 \pm 1.2$	7	5.3 ±0.8	7	0.001	0.01	0.01
	С	IFNy PEF+RT							
	D	IFNγ PEF+RT	$3.6 \pm 0.6$	7	$6.9 \pm 0.6$	7	0.001	0.35	0.004
All		IFNγ PEF+RT	$2.6 \pm 0.7$	14	6.12 ±0.5	14	0.0000003	0.0001	0.0001



#### Figure 3

Tumour growth rate in rats treated with tumours in all series-A, -B, -C, and -D with average of treated with either immunization, PEF, RT or (PEF+RT) and their combinations. The averages of the right lateral treated tumours are displayed in red columns. The tumour growth rate results of the corresponding untreated left-lateral tumours are displayed in green.

# B. Specific Therapeutic Effect (STE) and Specific Abscopal Effect (SAE)

In order to estimate the therapeutic effect we used the quantity "specific therapeutic effect" STE and the abscopal effect with the corresponding quantity "specific abscopal effect" SAE. The therapeutic effect is equivalent to the difference in tumour growth rate between the average of the controls and the exposed tumours relative to the tumour growth rate of the controls, calculated from the day of treatment.

The corresponding quantity "specific abscopal effect" SAE is equivalent to the difference in tumour growth rate between the average of the controls of unexposed rats and the unexposed contra-lateral tumours relative to the growth rate of the controls, calculated from the day of treatment.

Since the STE and SAE values are normalized to the controls of each experimental series they are pooled and treated as one population. The results of the individual series are given in **Table 5** and the pooled data of series-B, -C and -D are displayed in **Figure 4**.

#### Table 5a

Specific Therapeutic Effect (STE) and Specific Abscopal effect (SAE) in each experimental series and groups of treatment

Experimental	Group of	STE		SAE		t-test				
Series	Treatment	Average SE Right	N	Average SE Left	Ν	Right vs 0	Left l vs. 0	Right vs. Left		
٨	DEE									
B	PEF	0 34+0 12	8	$0.18 \pm 0.05$	8 8	0.02	0.07	02		
C C	PEF	$0.37\pm0.12$ 0.27±0.16	9	$-0.03\pm0.10$	) 9	0.02	0.07	0.2		
D	PEF	0.30±0.08	8	$0.12\pm0.06$	5 8	0.01	0.005	0.1		
All	PEF	0.30±0.08	25	0.11±0.05	5 25	< 0.001	0.04	0.02		
	DT									
A	KI DT	0.42 + 0.00	0	0.24+0.04	r o	<0.0005	0.01	0.16		
В		$0.42 \pm 0.06$	8	$0.24\pm0.06$	5 8	<0.0005	0.01	0.16		
D	RT	$0.49 \pm 0.04$	7	$0.30\pm0.04$	4 7	< 0.0001	0.001	0.11		
All	RT	$0.46 \pm 0.04$	15	0.33±0.04	4 15	< 0.0001	< 0.0001	0.05		
А	PEF+RT									
В	PEF+RT	0.29±0.16	7	$0.26 \pm 0.03$	3 7	0.25	0.001	0.98		
С	PEF+RT									
D	PEF+RT	$0.42 \pm 0.05$	7	0.27±0.02	2 7	0.0001	0.00001	0.07		
All	PEF+RT	0.36±0.08	15	0.26±0.02	2 14	< 0.0005	< 0.0001	0.23		

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#### Table 5b

Specific Therapeutic Effect (STE) and Specific Abscopal effect (SAE) in each experimental series and groups of treatment after immunization 3 times with syngeneic IFN<sub>γ</sub> secreting cells.

Experimental	Group of	STE Average SE N Right		SAF	t-test			
Series	Treatment			Average SE Left	N	Right Left vs. 0 vs. 0		Right vs. Left
А	IFNγ							
В	IFNγ	$-0.11\pm0.16$	8	$-0.17\pm0.23$	8	0.53	0.5	0.70
С	IFNγ	$0.26 \pm 0.07$	9	$0.15 \pm 0.05$	9	0.02	0.1	0.16
D	IFNγ	$-0.02\pm0.29$	2	-0.27±0.18	1	0.98	0.7	0.69
All	IFNγ	$0.09 \pm 0.07$	19	$-0.01\pm0.09$	19	0.23	0.9	0.21
А	$IFN\gamma + PEF$							
В	$IFN\gamma + PEF$	-0.17±0.05	8	$-0.29\pm0.15$	8	0.02	0.10	0.42
С	$IFN\gamma + PEF$	$0.22 \pm 0.14$	9	$-0.13\pm0.12$	9	0.43	0.21	0.02
D	$IFN\gamma + PEF$	$0.09 \pm 0.07$	8	-0.13±0.06	8	0.15	0.57	0.09
All	IFNy + PEF	$0.07 \pm 0.07$	24	$-0.14 \pm 0.07$	24	0.36	0.07	0.01
А	$IFN\gamma + RT$							
В	$IFN\gamma + RT$	0.26±0.07	8	0.14±0.12	8	0.01	0.16	0.38
С	$IFN\gamma + RT$							
D	$IFN\gamma + RT$							
All	$IFN\gamma + RT$	$0.26{\pm}0.07$	8	0.14±0.12	8	0.001	0.28	0.49
А	$IFN\gamma + PEF+RT$							
В	$IFN\gamma + PEF+RT$	$0.80 \pm 0.15$	7	$0.39 \pm 0.09$	7	0.002	0.01	0.02
С	IFNγ + PEF+RT							
D	$IFN\gamma + PEF+RT$	$0.57 {\pm} 0.07$	7	$0.11 \pm 0.07$	7	0.0002	0.01	0.003
All	$IFN\gamma + PEF+RT$	0.70±0.08	14	0.33±0.06	14	< 0.0001	0.0001	0.0002



#### Figure 4

Specific therapeutic effect STE (red) and specific abscopal effect SAE (green) from pooled data of series-B, -C, and-D. The p values indicate the significance level from the t-test that STE and SAE are different from zero. N is the number of rats in each group of various treatments.

# D. Therapeutic (TER) and Abscopal Enhancement Ratio (AER)

The therapeutic enhancement ratio of the combined treatment is defined as the ratio of the specific therapeutic effect of the combined treatment and the sum of the independent treatments.  $TER = STE_{Experimental} / STE_{Independent}$ 

The abscopal enhancement ratio of the combined treatment is defined as the ratio of the specific therapeutic effect of the untreated tumours on rats with combined treatment of the left-lateral tumour and the sum of the corresponding independent treatments.

#### $AER = SAE_{Experimental} / SAE_{Independent}$

Therapeutic enhancement ratio (TER) and Abscopal enhancement ratio (AER) at combined treatment with PEF, RT and IFN $\gamma$  are given in **Figure 5.** The values are derived from the

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averages of STE and SAE from the experimental series-B, -C and –D given in **Table 5**. Various combinations of independent treatments with PEF, RT or IFN $\gamma$ ; (PEF+RT) and IFN $\gamma$ ; PEF and (RT + IFN $\gamma$ ); RT and (PEF + IFN $\gamma$ ), has been used for calculating the TER and AER values when all three treatment modalities were combined.



#### Figure 5

Therapeutic enhancement ratio (TER) and Abscopal enhancement ratio (AER) at combined treatment with PEF, RT and IFN<sub>γ</sub>. The values are derived from the averages of STE and SAE from the experimental series-B, -C and –D given in **Table 5**. Various combinations of independent treatments with PEF, RT or IFN<sub>γ</sub> have been used for calculating the TER and AER values when all three treatment modalities were combined.

# **IV. DISCUSSIONS AND CONCLUSIONS**

# A. Tumour Growth Rate (TGR)

In the diagrams of Figure 4a and b it is shown that the tumour volume at 20-30 days after inoculation follow 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 tumour growth rate of each individual tumour is estimated from the daily tumour volume measurements of each tumour. The results of the tumour growth rate thus obtained are summarized in **Table 4**.

The TGR averages of the right and left lateral tumours from all experimental series are given in **Table 4** and displayed in **Figure 3**.

The averages of tumour growth rate of the right-lateral treated tumour was significantly decreased for independent treatments with PEF (p<0.005) in 25 tumours and RT (p<0.0001) in 15 tumours and with the combined treatment PEF+RT (p<0.005) in 15 tumours.

With immunization (IFN $\gamma$ ) alone in 19 rats and in combination with PEF in 24 rats there was, however, no significant decrease of the TGR of the right lateral tumours. But in the combination of immunization with RT in 8 rats and (PEF+RT) in 14 rats there was a highly significant decrease of the TGR values (p<0.001).

The tumour growth rate of the left-lateral untreated tumour in the groups with independent treatment of right-lateral tumours with PEF, there was no significant reduction of the tumour growth rate of the left-lateral tumours. But the TGR values are significantly reduced in the group of rats treated with RT (p<0.0001) in 15 tumours and the combination PEF + RT (p<0.0001) in 15 tumours. This effect on untreated tumours outside the target of treatment is called the "Abscopal effect".

With immunization (IFN $\gamma$ ) alone in 19 rats and in combinations with PEF in 24 rats and RT in 8 rats there was no significant decrease of the TGR in the left lateral tumours. But in the combination of immunization (IFN $\gamma$ ) with (PEF+RT) in 14 rats there was a highly significant decrease of the TGR values in the left lateral tumours. Thus in treatment with immunization the abscopal effect is significant only with the combined PEF+RT treatment.

This might indicate that PEF enhance the immune suppression by the tumour although the release of tumour specific proteins in the extracellular space. Radiation therapy, however, decrease the immune suppression by the tumour and then the tumour specific proteins induce an immune response that affect the left lateral tumour as well. This is in agreement with previous findings that cellular immunization give no anti-tumour response on tumours that produce immunosuppressive factors ( Graf et al. 2002; Lumniczky et al. 2002).

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# B. Specific Therapeutic Effect (STE) and Specific Abscopal Effect (SAE)

The "specific therapeutic effect" STE is obtained by normalizing the difference in tumour growth rate between the right lateral controls and exposed tumours to the tumour growth rate of the right lateral controls. The SAE values were obtained by normalizing the difference in tumour growth rate between the left lateral controls and the unexposed tumour 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 5**. This quantity is independent of time and normalized to the tumour growth characteristics of the controls of each experiment. In **Figure 4** are displayed the averages of STE and SAE from each type of treatment.

## 1. Specific Therapeutic Effect (STE)

Treatments with PEF alone resulted in an average STE value of  $0.30\pm0.01$  that is significantly different from zero (p<0.01). Treatment with RT alone resulted an average STE value of  $0.46\pm0.04$  that is highly significantly different from zero with p-values < 0.0001. The STE values for RT and PEF were not significantly different (p<0.07).

The treatment with PEF+RT in combination resulted in an average STE value of  $0.36\pm0.08$  which also is highly significant different from zero with p-values < 0.001. The STE values for PEF+RT versus the STE values for PEF or RT were not significantly different.

For immunization alone with IFN $\gamma$  secreting tumour cells there is a small therapeutic effect with a STE value of 0.09±0.07 not significantly different from zero. Also for immunization in combination with PEF, the STE value of 0.07±0.07 is not significantly different from zero. Since the STE value for PEF alone was much higher (0.30±0.01), the immunization treatment seems to suppress the therapeutic effect of PEF.

In the combination of RT with immunization the STE value was  $0.32\pm0.01$ , that is significantly different from zero and significantly different for RT alone (p<0.05).

The specific therapeutic effect of adding immunization to (PEF+RT) in combination resulted in an unexpectedly high STE value of  $0.70\pm0.08$  that is highly significantly different from zero ( p < 0.0001) and for the STE for PEF+ RT without immunization (p<0.01).

#### 2. Specific Abscopal Effect (SAE)

The results of the specific abscopal effect SAE of the contra lateral untreated tumours in rats treated with PEF or RT are both significantly different from zero. For RT the average SAE value is  $0.33\pm0.04$  ( p<0.001), but for PEF treated animals it is only  $0.11\pm0.05$  (p<0.05) and they are significantly different (p< 0.01). The average SEA value for the treatment with PEF + RT in combination is  $0.26\pm0.02$  (p<0.0001) that is not significantly different form SAE for RT alone but significantly different from SAE for PEF alone (p<0.01).

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For immunization with IFN $\gamma$  secreting tumour cells there is no effect on the contra lateral tumour. The corresponding SAE value was -0.01±0.09 not significantly different from zero. Thus if the effect of both right and left tumours are considered there is no significant effect of immunization alone.

The abscopal effect of immunization combined with PEF is negative. Since there was a significant effect of PEF alone this might indicate that the combination with immunization suppress the therapeutic effect.

Immunization combined with RT result in a SAE value of  $0.18\pm0.12$  which is much lower than the abscopal effect of RT alone. But due to the large variance of the SAE is not significantly different from zero (p=0.28) and for RT alone.

The combination of immunization with PEF+RT results in a SAE value of  $0.33\pm0.06$  that is slightly higher than for PEF+RT without immunization (0.26±0.02). That means that immunization combined with PEF+RT seems to improve the abscopal effect although the SAE values are not significantly different.

# C. Therapeutic (TER) and Abscopal Enhancement Ratio (AER)

The therapeutic enhancement ratio of the combined treatment is defined as the ratio of the specific therapeutic effect of the combined treatment and the sum of the independent treatments.

## TER = STE<sub>Experimental</sub> / STE<sub>Independent</sub>

The abscopal enhancement ratio of the combined treatment is defined as the ratio of the specific therapeutic effect of the left-lateral untreated tumours on rats at combined treatment of the right-lateral tumour and the sum of the corresponding independent treatments.

# AER = SAE<sub>Experimental</sub> / SAE<sub>Independent</sub>

The average of the therapeutic enhancement ratio (TER) and abscopal enhancement ratio (AER) from all experimental series are displayed in **Figure 5**. At combined treatment with PEF + RT the TER values is  $0.47 \pm 0.12$  and the AER value  $0.61 \pm 0.1$  respectively. It is interesting to note that the values Abscopal enhancement ratio (AER) are higher than the therapeutic enhancement ratio (TER). In a previous study of the therapeutic effect of PEF+RT on rat glioma the TER values were >1 in a few cases (Persson et al.2003c). In another recent paper the AER was >1 in one corresponding series (Persson et al. 2004). Thus by combining tumour treatment with pulsed electric fields and radiation therapy, PEF+RT there is an indication of that the treated tumour as well as on the distant non-treated tumours are affected more than additive.

By combining immunization with PEF alone the TER value is only about 0.2 and the AER is zero which means that there is no abscopal effect. But with all three treatment modalities combined and all combinations of independent treatments with PEF, RT or IFN $\gamma$  given if **Figure 5** are considered, the average of the TER is 1.20±0.15 and AER is 1.22±0.20. This might indicate that there is a synergism on the tumours on both sides by combining PEF, RT and immunization with IFN $\gamma$  secreting cells.

After PEF- treatment only there is no abscopal effect, while it is significantly increased after RT and

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PEF+RT treatment. This might indicate that RT produces specific factors responsible for the abscopal effect. Emerit et al. (1995) observed radiation-induced clastrogenic factors in plasma samples from RT - patients. It has also been observed that cells exposed with <sup>60</sup>Co  $\gamma$ -radiation produced a factor that mediates cell death in cells never exposed to radiation (Mothersill 2001). Other studies suggest that ionizing radiation induces the release of cytokines which mediate a systemic anti-tumour effect by activation of immune activity (Uchida 1989). The existence of radiation induced factors in vivo is now well accepted and they are likely to be tissue and patient specific (Mothersill 2002).

The mechanisms explaining this phenomenon might be either a molecular or an immunological effect which will be further considered in future investigations.

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