Immunobiol., vol. 180, pp. 261–271 (1990)

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Influence of Local Radiotherapy of Breast Cancer Patients on the Frequency of Cytotoxic T Lymphocyte Precursor Cells*

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Received March 13, 1989 · Accepted in Revised Form November 13, 1989

Abstract

Alloantigen-specific cytotoxic T lymphocyte precursor (CTL-p) frequencies were analyzed in ten patients with histologically proven breast cancer receiving prophylactic RT. The frequency of CTL-p was assessed by limiting dilution (LD) analyses before, immediately after discontinuation of treatment and at various times following RT. The number of pbmnc, adherent cells and T cells was determined in parallel.

Local RT led to a minor and transient reduction of CTL-p frequencies lasting approximately three months: on average a 25 % decrease of CTL-p numbers was seen immediately after RT. Three months following treatment, a 20 % reduction was still evident. Values subsequently returned to pretreatment levels. Moreover, these changes in the frequency of antigen-specific CTL were accompanied by a 25 % to 39 % decrease in the blood T cell counts lasting for more than 12 months. The reductions following local RT were less pronounced than those induced by immunosuppressive drugs in allograft recipients (1).

Introduction

The role of prophylactic postoperative radiotherapy (RT) in the treatment of breast cancer is still a matter of debate. The benefits arising from direct tumoricidal effect of irradiation on residual tumor cells may be counteracted by treatment-related immunosuppression negatively influenc-

*This work was financially supported by grants from the Austrian Research Fund «Zur Förderung der wissenschaftlichen Forschung», project no. 6526.

Abbreviations: Con A = Concanavalin A; *CTL* = cytotoxic T lymphocytes; *CTL-p* = cytotoxic T lymphocyte precursors; *Gy* = Gray; *IL-2* = recombinant interleukin 2; *LD* = limiting dilution; *pbmnc* = peripheral blood mononuclear cells; *RT* = radiotherapy; *SN* = supernatant.

ing the host's antitumor response (2). STJERNSWÄRD et al. (3) reported that radiotherapy in mastectomized patients leads to a long-lasting and marked lymphopenia, presumably caused by influencing host immunosurveillance of tumor cells. These authors also observed a decrease in blood T lymphocyte counts associated with a reduced *in vitro* proliferative response of blood lymphocytes to mitogenic stimulation with phytohemagglutinin (PHA). Subsequently, numerous investigators reevaluated the impact of radiotherapy on various immune parameters (3–6). Although much has been learned from these investigations about the influence of local RT on the number of T cells and their proliferative capacity, relatively little is known about the extent to which effector T lymphocyte numbers and/or functions are modified by such means. In the present study, we analyzed the influence of local RT on the frequency changes of cytotoxic T lymphocytes (CTL) in breast cancer patients. Results presented demonstrate that local RT causes a minor but measurable reduction of CTL-p frequencies.

Materials and Methods

Patients

Ten women with histologically verified stage I–III breast cancer receiving postoperative radiotherapy were included in this study. The median age was 53 years, ranging from 39 to 74 years. Details of their clinical characteristics are shown in Table 1. Results of LD analyses obtained from these patients were compared with those of healthy controls and patients undergoing surgery and are summarized in Table 1.

Table 1. Clinical findings of breast cancer patients

Patient	Age	Stage[a]	Surgical Intervention	Concomitant Therapy
OI	59	Gx, pT1 No Mo	partial mastectomy	none
KA	39	GII, pTx N1bMx	modified radical mastectomy	tamoxifen
BB	42	GII, pT2 No Mx	partial mastectomy	cortico-steroides
RH	54	GII, pT1 N1 Mx	modified radical mastectomy	tamoxifen
PO	65	GII, pT3 N1 Mx	partial mastectomy	tamoxifen
CS	48	GIII, pT2 No Mo	modified radical mastectomy	none
LP	52	GII, pT4 N1bMx	modified radical mastectomy	tamoxifen
TS	46	GII, pT1 No Mo	partial mastectomy	none
FA	62	GII, pT1 N1 Mo	partial mastectomy	tamoxifen
JA	74	GI, pT1 No Mo	partial mastectomy	tamoxifen
controls				
SR	62	goiter	strumectomy	
KAM	34	mastopathy	exploratory excision	
FG	45	GIII, pT2 N1bMo	partial mastectomy	

[a] Tumors were classified according to the grading (G) and the TNM system for breast cancer (UICC 1982) and were pathologically verified (p).

Treatment

Surgery

Four patients received a modified radical mastectomy and another six underwent partial mastectomy. Two to 16 weeks after surgery (median three weeks), routine postoperative RT was administered.

Radiotherapy

RT was performed using telecobalt or 8-MV photons from a linear electron accelerator. The irradiation technique employed tangential opposing vertical fields aimed at the residual breast or the chest wall including the parasternal region. In the majority of cases, computer-aided radiation planning was performed on an individual basis using CT images. In three patients, whose axillary lymph nodes were affected and in whom there was median tumor location, the supraclavicular region was also irradiated by a standing field. The total tumor dose for all irradiation fields was 60 Gy in the target area administered at a standard fractionation of 5 × 2 Gy per week. Six patients who had undergone conservative surgery received a booster of 10 Gy electrons to the former tumor site after an interval of ten to 14 days.

Laboratory Investigations

Separation of cells. Venous blood samples from breast cancer patients were collected before and at various intervals after RT. Peripheral blood mononuclear cells (pbmnc) were isolated from 50 ml heparinized blood by density gradient centrifugation on Lymphoprep (Nyegaard, Oslo, Norway). Pbmnc were frozen and cryopreserved in liquid nitrogen using previously published standard techniques (7). Adherent cells were depleted by incubating pbmnc in RPMI 1640 (Biochrom KG, Berlin, F.R.G.) with 10 % heat-inactivated human male pool serum (PS) in plastic Petri dishes (Falcon, Cockeysville, U.S.A.) for 90 min at 37 °C. T cells to be used as responders in limiting dilution cultures were isolated from non-adherent pbmnc by rosette formation with neuraminidase-treated sheep erythrocytes (E^+) using previously published standard techniques (8).

Limiting dilution (LD) analysis of CTL-p frequency. LD analyses were performed as previously published (9). Briefly, graded numbers (starting at 10,000 cells/well) of T responder lymphocytes were cocultivated in 24 replicates with a constant number (100,000 cells/well) of irradiated (20 Gy) allogenic pbmnc as stimulator cells in round-bottomed microtiter plates (Flow Laboratories, Meckenheim, F.R.G.). Stimulator cells were derived from two different unrelated donors. The HLA barriers between responder and stimulator cells tested are depicted in Table 2. Cultures were incubated for ten days at 37 °C in a 5 % CO_2, 95 % air atmosphere. The culture medium was RPMI 1640 supplemented with antibiotics, antimycotics, 2 mM L-glutamine, 10 % heat-inactivated human pool serum and 100 U/ml of recombinant interleukin 2 (IL-2, gift from Dr. E. Liehl, Sandoz Forschungsinstitut, Vienna).

Cultures were analyzed for cell-mediated cytotoxicity using the original stimulator cells as specific targets. Concanavalin A (Con A)-activated blasts from the stimulator cell donor were prepared by incubating 1×10^6/ml pbmnc for four days at 37 °C with complete culture medium supplemented with 20 µg/ml Con A (Difco, Detroit, MI, U.S.A.). Target cells ($2–3 \times 10^6$) were labelled for 120 min with 500 µCi ^{51}Cr (NEN-Chemicals, FRG; specific activity 5 mCi/ml). The cells were washed three times in the presence of 1-o-methyl-alpha-mannopyranoside (0.05 mM, SERVA, Heidelberg, FRG) to remove residual lectin. After removing 70 µl of supernatant (SN) from each well, 100 µl of ^{51}Cr-labelled Con A blasts were added to the microwells. Following an incubation period of four hours at 37 °C, 100 µl of SN were harvested and assessed for radioactivity in a well-type gamma counter (LKB, Wallac, 1272, Clinigamma, Finland) linked to a personal computer (IBM, XT). Maximum release was evaluated after vigorously resuspending target cells. Spontaneous ^{51}Cr-release was determined from wells containing only stimulator cells. The percentage of specific lysis was calculated as:

$$\% \text{ specific lysis} = \frac{cpm_{exp} - cpm_{spont}}{cpm_{max} - cpm_{spont}} \times 100$$

Table 2. HLA types of responder and stimulatory/target cells used in this study

Patients		Stimulator/ Target 1	Stimulator/ Target 2
OI	A 24 (A 9), A 25 (A 10) B 18, B 35, Bw 6 Cw 4	A 1 B 8, Bw 6 Cw 7	A 2, A 32 B 7, B 13
KA	A 24 (A 9) B 18, Bw 6	A 1 B 8, Bw 6 Cw 7	A 2, A 32 B 7, B 13
BB	Aw 19: 30+31, A 32 B 14, B 40, Bw 6	A 1 B 8, Bw 6 Cw 7	A 2, A 32 B 7, B 13
RH	A 2, Aw 19 B 7, B 35, Bw 4, Bw 6 Cw 4, Cw 7	A 1 B 8, Bw 6 Cw 7	A 2, A 32 B 7, B 13
LP	A 24 (A 9), A 11 B 5, B 39 (16), Bw 4, Bw 6 Cw 3	A 2, A 3 B 13, B 18	Aw 24, Aw 32 B 18, B 40 Cw 2
CS	A 2 B 18, B 27, Bw 6 Cw 2	A 1 B 8, B 40, Bw 4, Bw 6 Cw 2	A 2, A 24 (9) B 35 Cw 4
TS	A 2, A 3 B 7, Bw 62 (15), Bw 6 Cw 2	A 2, A 3 B 13, B 18	Aw 24, Aw 32 B 18, B 40 Cw 2
JA	A 1, A 28 B 17, B 40, Bw 4, Bw 6 Cw 3	A 1, A 3 B 7, B 14	A 2, A 11 Bw 62 (15), Bw 6 Cw 3
PO	A 2, A 11 B 7, B 40?, Bw 6 Cw 2	A 1 B 8, B 40, Bw 4, Bw 6 Cw 2	A 2, A 24 (A 9) B 35 Cw 4
FA	A 1, A 2 B 18, B 37, Bw 4, Bw 6	A 3, Aw 19 (w 31) B 15, Bw 16 (w 38) Cw 3	
Controls			
SR	A 2, A 26 (10) B 5 or Bw 53, Bw 41, Bw 4, Bw 6 Cw 4	A 24 B 7, Bw 44	A 3, Aw 19 (w 31) Bw 16 (Bw 38) Cw 3
FI	A 2, A 30 (Aw 19) B 13, B 44 (B 12), Bw 5 Cw 5	A 24 B 7, Bw 44	A 3, Aw 19 (w 31) Bw 16 (Bw 38) Cw 3
KAM	A 2, A 28 B 38 (B 16), Bw 22, Bw 4, Bw 6	A 24 B 7, Bw 44	A 3, Aw 19 (w 31) Bw 16 (Bw 38) Cw 3
MB	A 2, A 3 B 7, B 44 (12), Bw 4, Bw 6 Cw 7	A 2, A 9 B 13, B 27 Cw 2	A 11, Aw 19 Bw 44 (12) Cw 4
EI	A 28, A 24 (A 9) B 44, (B 12), Bw 4 Cw 5	A 2, A 9 B 13, B 27 Cw 2	A 11, Aw 19 Bw 44 (12) Cw 4

Specificity testing of CTLs generated under the above LD conditions was performed in two ways: Firstly, in two experiments IL-2 concentrations ranging from 50 to 200 U/ml were applied. Effector cells were then tested in parallel against the specific target and autologous Con A blasts. Self-killing was not seen at IL-2 concentrations below 200 U/ml but was a regular feature of cultures supplemented with higher amounts of IL-2. Secondly, split well analyses of cultures supplemented with 25 and 50 U IL-2/ml were performed in eight experiments. Results of these experiments indicated lack of self-killing with an occasional crossreactivity against MHC mismatched third-party targets (1, 9, and unpublished results).

Statistical analysis

Frequencies of CTL-p were calculated using the x^2 minimization procedure. Cultures with lysis values greater than the mean spontaneous release plus three standard deviations were considered positive and used to calculate CTL-p frequencies. The probability of single-hit conditions and the definition of the 95 % confidence intervals were determined according to statistical methods published by TASWELL (10).

Results

Influence of local RT on mononuclear blood cell counts

The numbers of various populations of mononuclear blood cells were determined in parallel with LD analyses of CTL-p frequencies. Results are shown in Table 3. Local irradiation in these patients led to a considerable reduction of absolute pbmnc counts lasting almost nine months. This reduction involved both E^+, non-adherent T cells and E^- adherent cells. Reduction of T cells was more pronounced and normalization was not reached by the end of this study (12 months after RT).

Influence of local RT on the frequency of CTL-p

The impact of local RT on the frequency of CTL-p was also analyzed. To define changes of alloantigen-specific CTL-p, frequencies were evaluated in

Table 3. Influence of local RT of breast cancer patients on mononuclear blood cell counts

Cell counts ($\times 10^6$/ml, means ± SD)								
	pbmnc			E^+lymphocytes			adherent cells	
breast cancer patients (n = 10)								
pre RT	1.23 ± 0.71	*		0.57 ± 0.36	*		0.32 ± 0.22	
after RT	0.86 ± 0.45	*	(−31 %)	0.35 ± 0.20	*	(−39 %)	0.24 ± 0.15	(−25 %)
3 mos	0.81 ± 0.44	*	(−25 %)	0.40 ± 0.30		(−30 %)	0.19 ± 0.14	(−41 %)
6 mos	0.77 ± 0.30	*	(−38 %)	0.38 ± 0.19	*	(−34 %)	0.31 ± 0.26	(− 4 %)
9 mos	1.10 ± 0.46		(− 2 %)	0.43 ± 0.18		(−25 %)	0.35 ± 0.21	(+ 9 %)
surgical controls (n = 3)								
preop.	2.03 ± 0.67			1.04 ± 0.61			1.05 ± 0.79	
postop.	2.01 ± 0.90		(− 1 %)	1.28 ± 0.75		(+23 %)	1.17 ± 1.41	(+11 %)

*$p < 0.05$

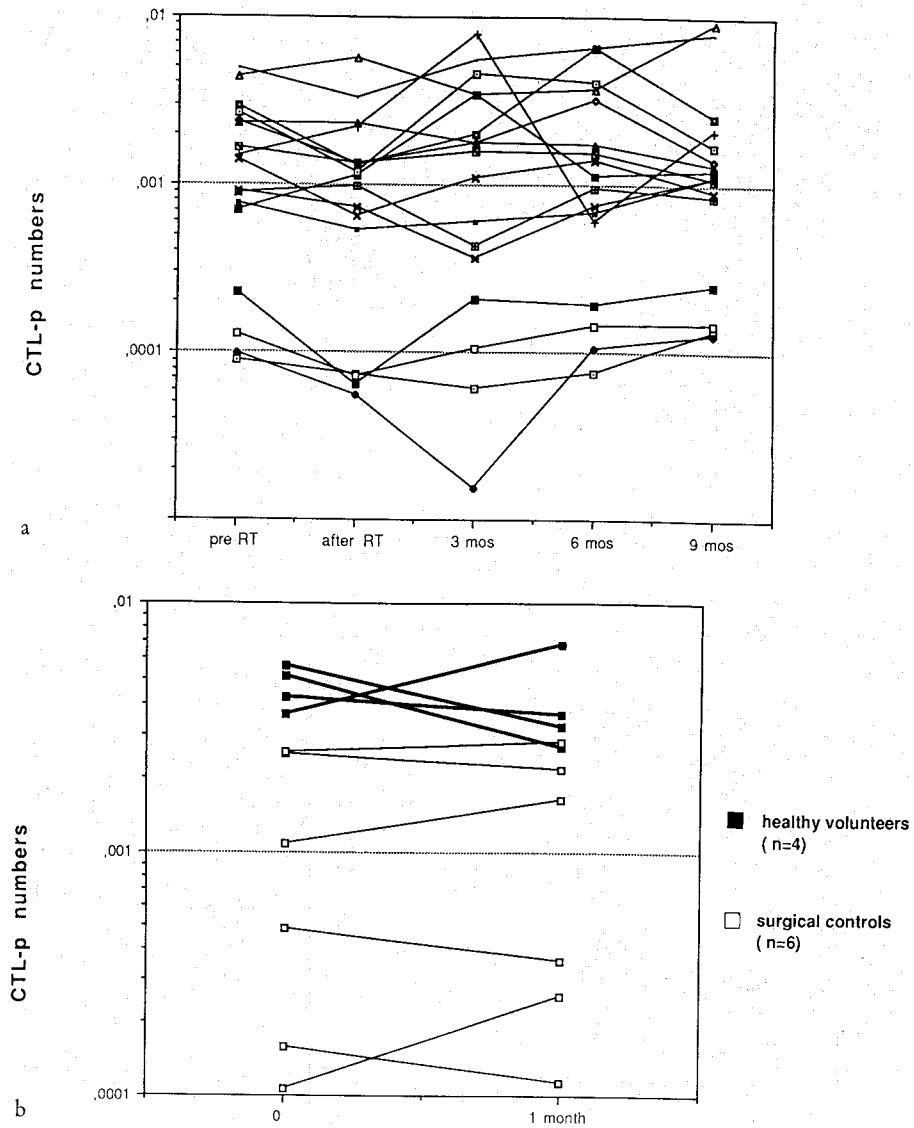

Figure 1. (a) A time course kinetics of alloreactive CTL-p from breast cancer patients prior to and immediately after RT and 3, 6 and 9 months later. Each symbol denotes a separate patient. (b) Frequencies of CTL-p of healthy volunteers (■) and surgical controls (□) at two different time points.

parallel against two different leukocyte donors. The results of the CTL-p numbers of patients and controls during the entire follow-up period are shown in Figures 1a and b. Both groups (patients and controls) exhibit a marked heterogeneity of alloreactive CTL-p frequencies ranging from 1 in 126 to 1 in 64,775 T cells. The individual patterns, however, remained remarkably stable throughout the entire period of investigation. Moreover,

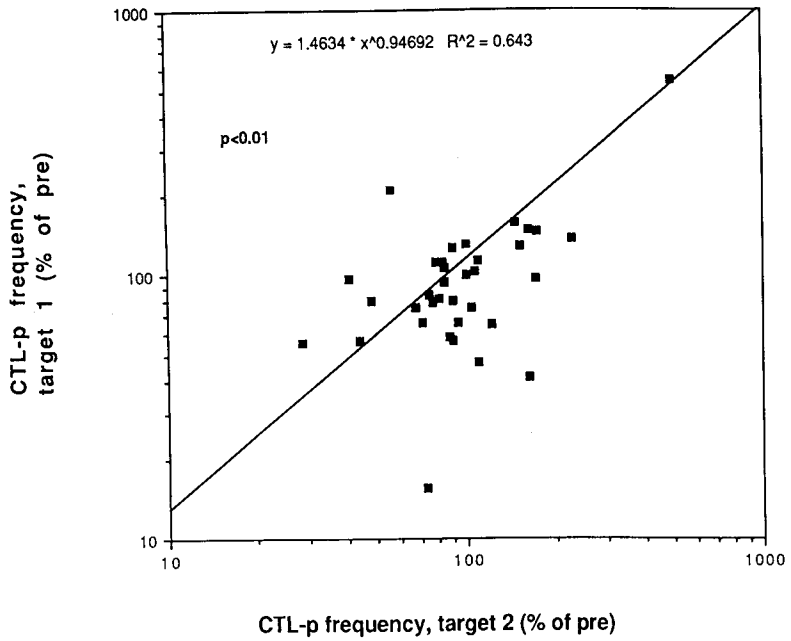

Figure 2. Correlation of RT-induced changes of alloreactive CTL-p frequencies. All frequencies are normalized by setting the pre-RT values to 100 %.

the minor changes of alloreactive CTL-p involved both specificities tested in parallel (Fig. 2). In order to facilitate evaluation of the impact of local RT or surgery on CTL-p frequencies, data were normalized by setting the individual pretreatment values at 100 % (Fig. 3). As indicated, local RT had

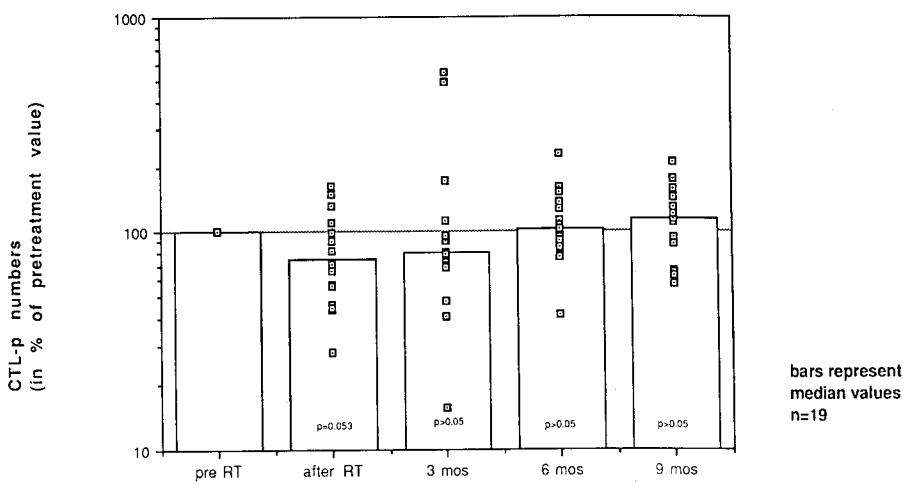

Figure 3. Frequencies of alloreactive CTL-p of breast cancer patients in percent of pretreatment values at various times before and after RT. Bars represent median values (n = 19).

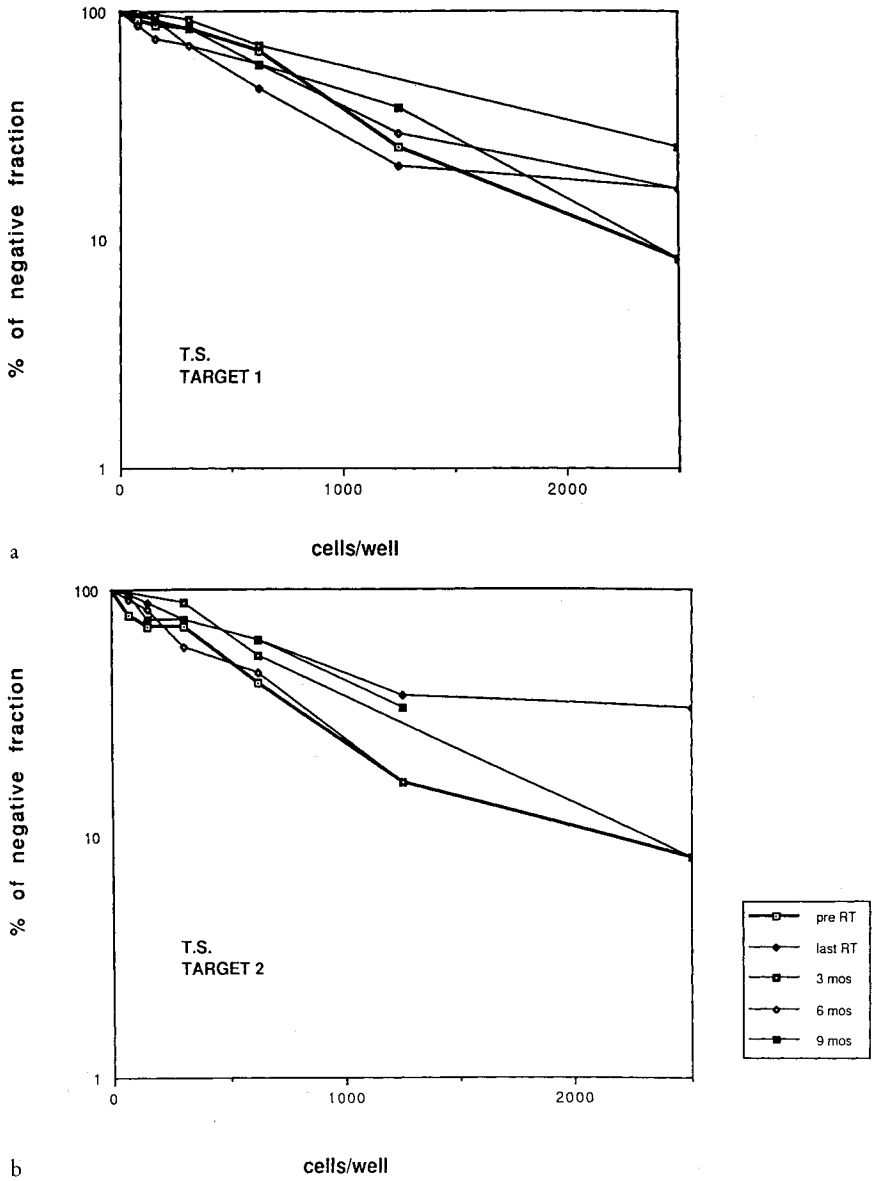

Figure 4. Representative plots of LD analyses of alloreactive CTL-p frequencies of breast cancer patient (T.S.) before (□), after local RT (◆), three (■), six (◆) and nine months (■) later, tested against two different leukocyte donors – target 1 (a) and target 2 (b).

only a minor and transient influence on CTL-p numbers. On the average, a 25.2 % median reduction (range: −71.8 % to +62.1 %) was seen immediately and a 20.1 % reduction (range: −84.5 % to +450 %) three months after RT. Values subsequently returned to or exceeded pretreatment figures.

Lack of evidence for induction of suppressive mechanisms by local RT

The patterns of LD plots permit conclusions to be drawn on the involvement of suppressive mechanisms. Evidence of suppressive influence is based on non-linear plots. Analyses of the breast cancer patients at various times before and after RT failed to support such a view. Representative LD plots are shown in Figures 4a and b. In a total of 87 of 111 individual analyses, single-hit conditions were observed as evidenced by linear plots, thus ruling out the involvement of suppression.

Discussion

The control of malignant growth by host immune cells, although not objectively proven in man, is nevertheless possible. Thus, antineoplastic therapies, which interfere with immunosurveillance, carry the potential risk of enhancing tumor spread. This view has prompted an intensive investigation of the various anticancer treatment modalities, including RT, for their impact on different immune parameters. The aim of this paper was to analyze the impact of local RT on the efferent limb of T cell immunity, which is crucial for the elimination of «altered self». LD studies of alloreactive CTL-p formed the test system chosen to obtain information about the frequency changes.

Our results confirm those of other authors demonstrating leukopenia subsequent to local RT (3, 4). In particular, a long-lasting impairment of T cell counts was observed. The extent and duration of RT-induced leukopenia were less pronounced in our series than those in previously reported studies (3). We assume that the improvement of radiation techniques is responsible for this difference. Changes of alloantigen-specific CTL-p frequencies were subject of particular study. Responses against two different allogenic targets were investigated in parallel. Local RT of breast cancer patients had a minor and transient influence on the relative frequency of alloreactive CTL-p. Both antigenic specificities exhibited identical changes in their frequencies subsequent to RT. We take this as evidence that these changes reflect the overall response modification of the host's CTL-p repertoire. To what extent helper T lymphocytes are influenced by prophylactic RT is still unknown. Recent data suggest that, at least after total lymphoid irradiation, there is a generation of suppressor cells responsible for induction of tolerance (11). We used LD analyses to address this question. It has been demonstrated that the involvement of suppressive mechanisms, including suppressor cells, influences the patterns of LD plots. In these cases, LD plots were shown to follow bell-shaped curves, indicating «multi-hit» conditions rather than linear plots characteristic of «single-hit» conditions (12). The LD analyses of breast cancer patients subsequent to local RT satisfied the above mentioned prerequisites for «single-hit» conditions. These studies thus failed to support the view that generation of

RT-induced T suppressor cells is the mechanism underlying clonal reduction of CTL.

It is not easy to discuss our results in context with previously published LD data. It is rather difficult to compare LD assays of different groups only under the aspect of the IL-2 concentrations used, because these assays are performed in multiple variations. IL-2 differs in concentration and source. Concentrations of 200 U IL-2/ml were used by ERARD et al. (13) in a mouse system. HERZOG et al. used 50 U IL-2/ml (1), KABELITZ et al. 25 U IL-2/ml (9), whereas concentrations of 5 U IL-2/ml on days three and six were given by KAMINSKI et al. (14). The culture period of ten days was comparable in all reports, whereas the purity of the responding cells was quite different. Some authors used pbmnc as responder cells (14), while others tested purified T cells (1, 9). The LD assay also differed in the number of stimulating cells: KABELITZ et al. and HERZOG et al. used 1×10^5 pbmnc and KAMINSKI et al. 5×10^4 pbmnc per culture. Target cells were either $1–2 \times 10^3$ Con A blasts (1, 9) or 1×10^4 PHA blasts per well (14).

The significance of demonstrating a minor reduction in CTL-p frequencies subsequent to local RT of cancer patients is unknown. It has been shown that both conventional immunosuppressive therapy and cyclosporin A treatment of allograft recipients lead to a marked reduction of the donor-specific CTL-p frequencies (1). This reduction was more than 50 % on average and was thus much more pronounced than the RT-induced changes observed in the present study. A 50 % decrease of donor-specific CTL-p subsequent to immunosuppressive therapy was invariably associated with a state of acquired allograft tolerance. Our conclusion is that changes in CTL-p frequencies observed after local RT are less pronounced and not comparable to the severe impairment of the efferent limb of T cell immunity seen during pharmacological immunosuppression. Although the antigenic specificities studied do not directly relate to the problem of tumor cell immunosurveillance, we assume that they relate to the efferent side of the host's immune system.

Acknowledgements

We are grateful to Mr. WERNER KLOTZ and Dr. JOHANN KAMLEITNER for skillful technical assistance.

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