

In vivo modulation of natural killer cell activity by tamoxifen in patients with bilateral primary breast cancer

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Abstract. Natural killer (NK) cell activity, the autologous mixed lymphocyte reaction (AMLR) and proportions of T cell subpopulations (CD3⁺/CD4⁺ and CD3⁺/CD8⁺) and NK cells (CD16⁺) were studied in 21 patients with bilateral primary breast cancer (BBC), 10 patients with single-breast cancer (SBC) and 20 healthy controls. All patients studied had no evidence of disease and had been off radiotherapy and/or chemotherapy for at least 1 year. Ten patients with BBC were also treated with tamoxifen. Patients with SBC had NK cell activity, AMLR responses and T cell subpopulations that were comparable to those of normal controls. In patients with BBC, a significant ($P < 0.01$) increase in NK activity compared to that in normal controls ($42 \pm 13\%$ versus $21 \pm 10\%$, effector-to-target cell ratio, 25:1) and a significant ($P < 0.05$) decrease in CD4⁺ T cell proportions ($30 \pm 15\%$ versus $49 \pm 13\%$) and absolute numbers ($472 \pm 82/\text{mm}^3$ versus $953 \pm 131/\text{mm}^3$) were found. However, the proliferative response of BBC patients' T lymphocytes in AMLR was in the range of the normal controls. Lymphocytes derived from 10 BBC patients treated with tamoxifen exhibited NK cell activity that was comparable to that of normal controls and patients with SBC, and was significantly ($P < 0.01$) reduced compared to the pretreatment period. BBC patients who received tamoxifen also show a reduction in the proportion of CD4⁺ T cells and in AMLR proliferative responses, which decreased compared to levels in normal controls. Taken together, these results indicate that long-term tamoxifen treatment modulates immune responses in BBC patients.

Key words: NK activity – Tamoxifen – Breast cancer – Multiple primary neoplasms

Introduction

Patients with breast cancer have a threefold increase in the risk that a second breast cancer will develop [6, 9]. Although numerous studies have dealt with in vitro immune functions in single-breast cancer [21, 22], to the best of our knowledge no study of immune responses in patients with bilateral primary breast cancer (BBC) has been published.

There is evidence that sex hormones, both male and female, may influence immunological functions [13]. Clinical trials have shown that adjuvant treatment with tamoxifen, a non-steroid drug with estrogenic and anti-estrogenic effects, can prolong disease-free and overall survival in patients with breast cancer [10, 14]. The antitumor activity of tamoxifen is not exclusively due to binding to estrogen receptors on tumor cells. Other mechanisms, like an effect on the natural immune defense against cancer, may contribute to the clinical activity of tamoxifen [11].

In the present investigation we studied immune functions in BBC patients and examined whether adjuvant long-term tamoxifen treatment modulates the activity of in vitro immune responses. Our results show high natural killer (NK) cell activity in BBC patients, which is modulated together with lymphocyte proliferation and CD4⁺ T cell proportion in patients treated with tamoxifen.

Materials and methods

Patients. A group of 21 women with bilateral metachronous breast cancer (BBC) and 10 women with single-breast cancer (SBC), all with no evidence of disease, was studied. Metachronous tumors were defined as those appearing in the contralateral breast within an interval of 6 months or more after the first cancer. The age range of the BBC group was 31–70 years (mean 53) and that of the SBC group 37–87 years (mean 55). Ten patients with BBC were subjected to prolonged oral treatment with tamoxifen, 20 mg twice daily, while being off radiotherapy and/or chemotherapy for at least 1 year. Patients were checked clinically at least every 3 months.

Separation of lymphoid cells. A 20-ml sample of venous blood was drawn from each individual into a heparinized syringe. Mononuclear

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cells were obtained by using the standard fractionation of blood on a Ficoll/Hypaque gradient. Helper and suppressor cells were identified by employing three murine monoclonal antibodies (Ortho Pharmaceuticals, Raritan, N. J.): OKT3 for mature CD3⁺ T lymphocytes, OKT4 for helper CD4⁺ T cells and OKT8 for suppressor/cytotoxic CD8⁺ T cells. NK cells (CD16⁺) were identified by the monoclonal antibody Leu11b (Becton-Dickinson, Mountain-View, Calif.). The appropriate monoclonal antibody was added to aliquots of 1×10^6 mononuclear cells, which were then stained with fluorescein-isothiocyanate-conjugated goat anti-mouse IgG antibody (Tago, Burlingame, Calif.) and read in a FACScan (Becton-Dickinson).

NK activity of lymphocytes. NK cell assay was performed as previously described [8]. Patients were assigned to groups of 2 or 3 of similar age. A healthy subject with the same characteristics of sex and age was matched to each group, and the test was performed the same day for the patients and their matched control. Mean percentage lysis was calculated by the following formula:

$$\text{Lysis (\%)} = \frac{\text{experimental } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}}{\text{maximal } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}} \times 100$$

Autologous mixed lymphocyte reaction (AMLR). Mononuclear cells were rosetted with sheep red blood cells. After overnight incubation in 4°C and fractionation on Ficoll/Hypaque gradients, two populations were obtained: T cells (rosetting) and non-T cells (non-rosetting). Non-T cells, which served as stimulator cells in AMLR, were irradiated with 30 Gy and mixed with T cells (responder cells) at a 1:1 ratio. Cultures were incubated at a final cell concentration of 2×10^6 cells/ml for 6 days, pulsed with [³H]-thymidine for another 16 h and harvested. The degree of [³H]-thymidine incorporated into DNA was determined and expressed as counts per minute (cpm).

Results

Proportions and absolute numbers of T lymphocyte subpopulations are shown in Table 1. CD4⁺ T cell proportions were significantly lower in BBC patients compared to normal matched controls ($30 \pm 15\%$ versus $49 \pm 13\%$, $P < 0.05$). In tamoxifen-treated BBC patients, the proportions of CD4⁺ T cells were further reduced ($15 \pm 6\%$).

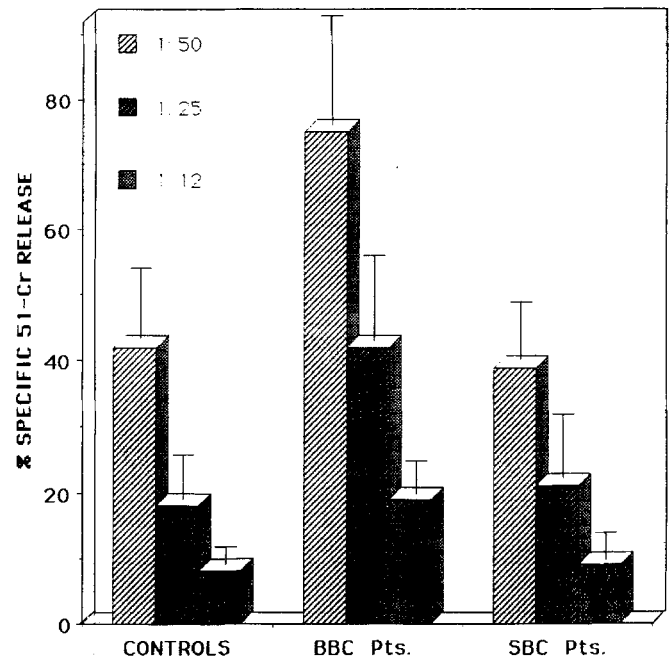


Fig. 1. NK activity of lymphocytes derived from patients with primary bilateral breast cancer (BBC), single-breast cancer (SBC) and normal controls. Lymphoid cells were purified from peripheral blood and cultured with ⁵¹Cr-labelled K-562 tumor cells at various effector-to-target cell ratios. Lytic activity has been calculated as described in Materials and methods

NK activity of BBC patients was significantly higher ($P < 0.01$) compared to SBC patients and normal matched controls ($42 \pm 13\%$, $21 \pm 10\%$, $18 \pm 7\%$ respectively, E:T 25:1) at all effector:target ratios tested (Fig. 1). However, the NK activity of 10 BBC patients who received adjuvant tamoxifen treatment was reduced and became comparable to that of normal controls ($25 \pm 9\%$ versus $21 \pm 10\%$, E:T 25:1) (Fig. 2). The proportions and absolute numbers of CD16⁺ lymphocytes in peripheral blood of patients either

Table 1. Lymphocyte subsets in tamoxifen-treated and untreated patients with bilateral primary breast cancer^a

Parameter	CD3	CD4	CD8	CD4/CD8	CD16
Lymphocytes from untreated patients (n = 13)					
Relative amount (%)	44 ± 14	30 ± 15	29 ± 10	1.2 ± 0.7	13 ± 4
Absolute no. (mm ⁻³)	793 ± 97	472 ± 82	457 ± 61		205 ± 16
Lymphocytes from tamoxifen-treated patients (n = 7)					
Relative amount (%)	33 ± 9	15 ± 6	31 ± 11	0.5 ± 0.3	15 ± 6
Absolute no. (mm ⁻³)	882 ± 86	373 ± 22	572 ± 73		273 ± 21
<i>P</i> ^b	<0.1	<0.05	NS	<0.05	NS
Lymphocytes from normal controls (n = 20)					
Relative amount (%)	67 ± 13	49 ± 13	32 ± 10	1.6 ± 0.6	14 ± 8
Absolute no. (mm ⁻³)	1302 ± 178	953 ± 131	522 ± 73		272 ± 25

^a Peripheral blood mononuclear cells were stained with the monoclonal antibodies OKT3 (CD3), OKT4 (CD4), OKT8 (CD8) Ortho and Leu11b (CD16) (Becton-Dickinson). The percentage of positive cells stained with a monoclonal antibody was determined by using the FACScan (Becton-Dickinson). All results are means ± SD

^b *P* denotes the statistical significance of the differences between positively stained cells of untreated patients and those of tamoxifen-treated patients

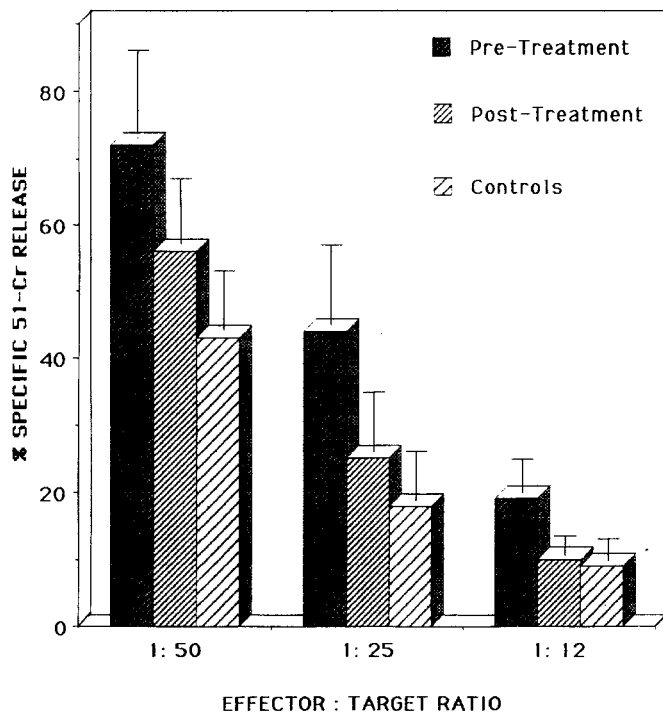


Fig. 2. Effect of tamoxifen treatment on natural killer (NK) activity of BBC patients. Lymphoid cells were derived from 10 BBC patients who received a prolonged treatment with tamoxifen, and NK activity was measured as described in the legend to Fig. 1

Table 2. Effect of tamoxifen treatment on T cell proliferation in autologous mixed lymphocyte reaction of patients with bilateral primary breast cancer (BBC)^a

Sample	[³ H]Thymidine incorporation	<i>P</i> ^a (<i>t</i> - test)
Untreated BBC patients (<i>n</i> = 10)	16498 ± 1916	
Tamoxifen-treated BBC patients (<i>n</i> = 10)	8567 ± 2221	<0.01
Healthy controls (<i>n</i> = 20)	13884 ± 2150	0.05

^a Mononuclear cells of 10 BBC patients treated with tamoxifen and 20 healthy controls were separated into T and non-T cells and cultured for 7 days as described in Materials and methods. Proliferation of T cells in cultures was evaluated by incorporation of [³H]thymidine into the proliferating cells

^b *P* denotes the statistical significance of the differences between [³H]thymidine incorporation by T cells of non-treated BBC patients and that of tamoxifen-treated patients or healthy controls

treated or untreated were comparable to those of normal controls (Table 1).

Proliferation of T lymphocytes in AMLR studied in BBC patients was in the range of that seen in normal controls and SBC patients (16498 ± 1916, 15884 ± 2150, 19298 ± 3872 respectively, *P* = 0.05). However, BBC patients who received tamoxifen showed significantly reduced (*P* < 0.01) proliferative responses of T cells compared to normal controls (Table 2).

Discussion

We had two goals in our investigation: to study immune functions in patients with BBC and to evaluate the possible immunomodulatory effect of tamoxifen. The results of our study show that patients with BBC have significantly increased NK activity, which is modulated by long-term tamoxifen treatment.

Studies of NK cell function in patients with SBC have shown cytotoxic activity comparable to that in normal controls [2, 4, 7] as was also found in our study. In these studies it made no difference whether patients were untreated or surgically treated or whether disease was localized or disseminated. Investigators also found scarcely reduced NK activity in women with SBC [23]. However, studies done in SBC patients who had received chemotherapy generally show reduced NK activity [3, 5, 12, 19]. Against this background, our finding of markedly increased NK cell activity in BBC patients with no evidence of disease is of special interest and may suggest in vivo activation of immune surveillance mechanisms in these patients. Alternatively, increased NK activity may result from viral stimulation [18], thus suggesting involvement of viral activity in patients with multiple primary breast tumors. AMLR is an in vitro test reflecting in vivo immunoregulatory circuits [16]. The main population of cells that undergo proliferation in this test are the CD4⁺ T cells. Normal proliferative responses of lymphocytes derived from BBC patients in AMLR, as was found in our study, in the face of reduced numbers of CD4⁺ cells, may point to overproliferation of CD8⁺ suppressor/cytotoxic T cells. This may also support the notion that in vivo cytotoxic immune responses are activated in BBC patients.

Tamoxifen is an antiestrogen that competes for estrogen receptors. It has established itself as an effective form of hormone therapy in estrogen-receptor-positive breast cancer patients and as an adjuvant treatment in a metastatic setting. Sheard et al. [19] found that in patients who received tamoxifen for 1 week there was an increase in total T lymphocyte and T-helper cell counts with a less marked change in T-suppressor/cytotoxic cells. Berry et al. [1] tested 17 postmenopausal women who suffered from SBC and were treated with tamoxifen for 1 month. A statistically significant increase in NK cell activity was demonstrated. However, Rotstein et al. [17] who examined patients treated with tamoxifen for 1.5–2 years, found significantly lower NK activity as compared to healthy controls. As all of our patients had prolonged tamoxifen treatment, our results are in accordance with the findings of Rotstein et al. It is not clear from our study whether the effect of tamoxifen is direct or through its antiestrogen activity.

Regarding lymphocyte subsets, we previously found that in BBC patients with no evidence of disease, CD4⁺ lymphocyte proportions and the ratio CD4/CD8 were significantly decreased as compared to normal controls but were in the same range as those of patients with SBC [15]. In the present study, further significant reduction in CD4⁺ proportions and CD4/CD8 ratio was seen in BBC patients who received adjuvant tamoxifen treatment.

In vitro tests of NK cell activity and lymphocyte proliferation in AMLR may reflect in vivo immunosurveillance mechanisms. For instance, low NK cell activity may predict early recurrence of neoplastic disease [20]. We found that long-term tamoxifen treatment lowers NK activity and lymphocyte proliferation in AMLR. Thus, further investigation is warranted to determine the role of long-term tamoxifen treatment in modulation of immune responses in BBC patients.

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