Acta Radiologica Oncology 24 (1985) Fasc. 2

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BLOOD LYMPHOCYTE POPULATION 4 TO 6 YEARS AFTER ADJUVANT CYCLIC CHEMOTHERAPY FOR OPERABLE BREAST CARCINOMA

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Abstract

The influence of postoperative cyclic chemotherapy for breast carcinoma on the blood lymphocyte population was examined in 21 recurrence-free women 4 to 6 years after completion of therapy. Chemotherapy consisted of combinations of chlorambucil, methotrexate and 5-fluorouracil or cyclophosphamide, methotrexate and 5-fluorouracil. It was observed that the lymphocyte numbers and mitogenic responses to PHA and in an MLC, known to be depressed during and shortly after treatment, had recovered to the levels of healthy age-matched women. NK activity, which is increased during the period of chemotherapy, still remained elevated.

The disease-free survival of patients with relatively advanced primary breast carcinoma may be prolonged by local radiation therapy or cyclic chemotherapy given as adjuncts to surgery (4, 20). In an ongoing trial in the Stockholm region the clinical value of postoperative local radiation therapy is compared with that of cyclic chemotherapy in breast cancer patients at high risk of developing recurrent disease. In the beginning of the trial the patients received a combination of chlorambucil, methotrexate and 5-fluorouracil (LMF chemotherapy). Later, however, chlorambucil was replaced by cyclophosphamide (CMF-therapy).

In previous investigations we observed that these chemotherapy regimens reduce the blood lymphocyte counts to approximately the same extent and various rosette tests which were used to identify T- and non-T-lymphocytes suggested that the latter were depleted to a somewhat higher extent by both regimens (19). The relative mitogenic responses of the lymphocytes to phytohaemagglutinin (PHA) and in a mixed lymphocyte culture (MLC) were reduced during and shortly after completion of therapy (19). Interestingly, the relative natural killer (NK) cell activity against a cultured cell line termed Chang increased during the entire period of CMF-therapy and remained significantly elevated 7 months after its completion (3).

The aim of the present investigation was to examine the long-term effects of adjuvant cyclic CMF and LMF therapy on the blood lymphocyte population. For this purpose recurrence-free patients who had completed their treatment 4 to 6 years earlier were compared with age matched healthy women who were tested in parallel.

Materials and Methods

Patients and controls. The investigation comprised 21 patients and 11 healthy controls. The patients were all women who had previously received postoperative LMF or CMF therapy for primary breast tumors exceeding 3 cm in diameter and tumors with histopathologically confirmed malignant involvement of axillary nodes. The patients had remained disease-free after treatment and were clinically disease-free at the time of the tests. Their ages ranged from 39 to 73 years with a mean of 56.

Accepted for publication 11 May 1984.

Eleven of the patients had received LMF treatments which were completed 60 to 76 months before testing and 10 of the patients had received CMF treatments 47 to 59 months earlier. Two of the latter patients had also been treated with Nolvadex (Tamoxifen) during a 2-year period. The mean time period which had elapsed since chemotherapy was 60 months for both patient groups.

Healthy female staff members served as controls. Their ages ranged from 38 to 64 years with a mean of 52.

Therapy of the patients. All the patients underwent a modified radical mastectomy with dissection of the axilla. Four to six weeks later chemotherapy was started. The LMF therapy consisted of 600 mg/m^2 of 5-fluorouracil and 50 mg/m^2 of methotrexate intravenously on days 1 and 8 and 10 mg/m^2 of chlorambucil orally on days 1 through 8. The next cycle was started on day 42. The CMF therapy consisted of 600 mg/m^2 of 5-fluorouracil and 40 mg/m^2 of methotrexate intravenously on days 1 and 8 and 100 mg/m^2 of cyclophosphamide orally on days 1 through 14. The next cycle was started on day 42. Each patient received a total of 12 cycles of LMF or CMF which were given during a period of approximately 17 months.

In most of the patients dose reductions were performed in one or more cycles due to bone marrow toxicity or other side effects. The extent of dose reductions have been described in detail before (19).

Separation of lymphoid cells. Lymphoid cells were separated from heparinized venous blood by centrifugation on a layer of Ficoll-Isopaque (5). The cells were washed twice by centrifugation in Eagle's Minimal Essential Medium supplemented with Earle's salts (MEM). These preparations of predominantly small lymphocytes, which were contaminated with approximately 10 per cent of monocytes, were used for MLC and PHA stimulation tests. Cell preparations which were used for rosette tests and NK activity were subjected to a purification step in which monocytes were depleted using iron powder and a magnet as described by BLOMGREN (1).

Rosette tests. Lymphocytes expressing membrane receptors (FcR) for the Fc-part of IgG were identified as cells forming rosettes with ox erythrocytes sensitized with specific antibodies (IgG) as described by PETRINI et coll. (14). These cells will be termed EA-rosette forming.

NK-cell activity. This assay has been described before (7). In short, lymphocytes were incubated with ^{51}Cr-labelled Chang cells (derived from human liver) and K562 cells (derived from a human myeloid leukemia) for 4 h at 37°C. The lymphocyte: target cell ratios ranged from 12.5: 1 to 100: 1. Cytotoxicity of the lymphocytes was calculated according to the following formula: (% isotope release with lymphocytes – % spontaneous release)/(100–% spontaneous release)×100.

Arithmetic mean values from duplicate tests were calculated.

Mitomycin treatment of cells. Approximately 5×10^6 cells were suspended in 3 ml of MEM containing 25 μg/ml of Mitomycin C (Sigma Chemical Co., St. Louis, USA). After 30 min at 37°C the cells were washed three times by centrifugation in MEM.

Lymphocyte stimulants. PHA and allogeneic lymphoid cells were used to induce DNA-synthesis in lymphocytes. The PHA preparation (Bactophytohaemagglutinin M, Difco Lab., Detroit, USA). The contents of vials containing PHA were dissolved in 5 ml of MEM (100% solution). Lymphoid cells were cultured with PHA at a final concentration of 3 per cent (v/v) which was observed to be optimally stimulatory. The allogeneic lymphoid cells, not depleted of monocytes, were obtained from the blood of 20 healthy subjects. The cells were pooled and stored in liquid nitrogen as described by WOOD et coll. (22) and JUHLIN et coll. (10).

Lymphocyte stimulation tests. These assays have been described before (11). In brief, lymphoid cells were cultured in the wells of plastic plates containing 0.2 ml of MEM supplemented with antibiotics and 10% of human heat inactivated AB+ serum. When testing the MLC response each well contained 2×10^5 responding lymphocytes and 2×10^5 Mitomycin C treated autologous or allogeneic lymphoid cells and 10^5 lymphocytes per well were used for PHA stimulation tests. Control cultures contained an equal number of cells without PHA. Cultures were set up in triplicate. They were incubated at 37°C in a humidified 5% CO_2-air atmosphere for 5 days. Twenty-four hours before termination each culture received 37 kBq of ^{3}H-thymidine (185 GBq/mmol, Amersham International, Amersham, England). Incorporated radiation activity, expressed as counts per minute (cpm), was determined using liquid scintillation counting. Arithmetic means of triplicate determinations were calculated.

Experimental design and statistical evaluation. Blood was drawn from women who had received

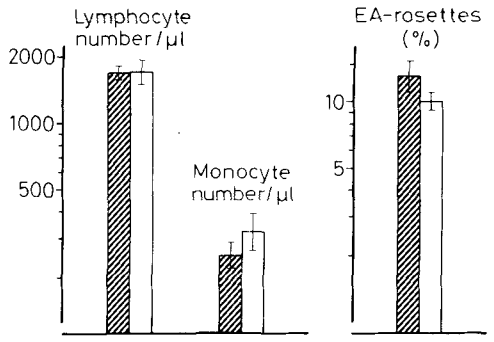

Fig. 1. Lymphocyte and monocyte counts per µl of blood and frequency of EA-rosette forming lymphocytes in patients treated with cyclic chemotherapy 4 to 6 years earlier (▨) and in healthy controls (□). M±SE are presented. Absolute cell numbers are calculated from 20 patients and 10 controls and EA-rosettes from 16 patients and 11 controls. Patients and controls did not differ significantly.

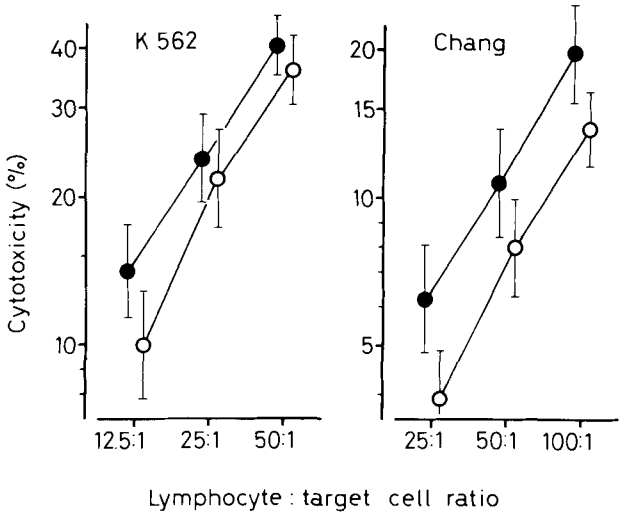

Fig. 2. NK activity of lymphocyte preparations from patients (●) and controls (○). Nineteen patients and 10 controls were tested against K562 cells and 14 patients and 9 controls against Chang cells. M±SE are shown. NK activity of the patients' lymphocytes against Chang cells was significantly higher than that of the controls ($p<0.05$ for all ratios).

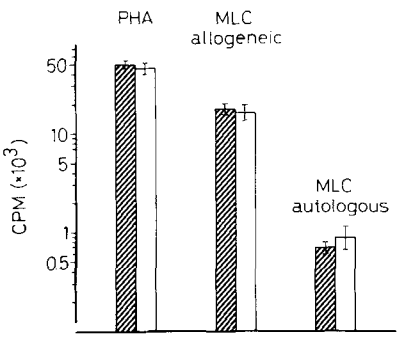

Fig. 3. ^{3}H-thymidine incorporations, expressed in cpm, of lymphocytes obtained from patients (▨) and controls (□). The cells were cultured with PHA or together with allogeneic or autologous lymphoid cells. Twenty-one patients were tested and 10 or 11 controls. M±SE are shown. Patients and controls did not differ significantly.

LMF or CMF therapy 4 to 6 years earlier. On the same day blood was also drawn from a healthy woman who had not been treated for any malignant disease. On each test occasion the blood from up to 3 patients and one control was tested.

For studies on the statistical significance of differences between patients and controls the results were processed in two ways. 1) The values of each patient were related to the corresponding values of the control person and expressed in per cent. 2) The arithmetic mean values of the patients who were tested on the same day were related to the corresponding values of the control person. Using the latter method the number of patient observations equalled that of the controls.

The mean values were calculated on a geometric basis and expressed in $\log_{10}$ units. Statistical significance was calculated using the Student's t-test.

Results

Fig. 1 shows that the mean lymphocyte and monocyte counts per µl of blood and percentages of EA-rosette forming lymphocytes in patients and controls were closely similar. Statistical evaluations of the results, according to the methods described, did not reveal any differences between the two groups.

Fig. 2 shows the NK activity of lymphocytes obtained from patients and controls for K562 and Chang cells. The NK activity of the patients' lymphocytes for the latter target cell was significantly higher than that of the controls ($p<0.05$ for all lymphocytes: target cell ratios). This difference was obtained when the results of the patients tested on the same day were pooled and related to the respective control subject. The mean NK activity against K562 cells was also higher in the patients than in the controls, but the difference was not statistically significant.

The PHA and MLC responses of lymphocytes from patients and controls are presented in Fig. 3. There were no significant differences between these two groups of individuals.

Discussion

Compared with local radiation therapy for breast carcinoma cyclic chemotherapy with LMF or CMF seems to alter the blood lymphocyte population to a relatively small extent (for a review of the radiation

induced changes, see 2 and 21). Most of these results are based on tests made during the 17 months long period of chemotherapy and shortly thereafter (3, 13, 17, 18). To our knowledge no investigations have previously been reported concerning long-term effects on the lymphocyte population of adjuvant cyclic chemotherapy in operated patients with breast carcinoma. However, several reports have dealt with such effects after other types of cancer chemotherapy (6, 8, 12, 16).

In this investigation we have examined the size of the blood lymphocyte population and some immunologic functions 4 to 6 years after completion of LMF or CMF chemotherapy. Since most of the assays used exhibit large day to day variations an age matched healthy control was always tested in parallel for comparison. The results showed that the lymphocyte counts and the MLC and PHA responses, known to be depressed during and shortly after the period of chemotherapy (19), were closely similar to those of the controls indicating a complete recovery. The NK activity of the lymphocytes for K562 and Chang cells seemed to be increased in the patients. Variability between the tests, however, was extensive and statistical significance was only reached for Chang cells when the data of the patients tested on one occasion were pooled and related to the concomitant control. It should be pointed out that all statistical calculations have been performed using logarithmized values which give a close to normal distribution of data in materials containing extreme values. If, however, the calculations are made on an arithmetic basis the differences in NK activity between patients and controls is more marked and is statistically elevated against both target cells. No other parameters studied turned significant using this method (data not presented). Our results suggest that the NK activity, which is increased during chemotherapy (3) remains elevated for several years. The most likely explanation is that this is due to a longstanding redistribution of cells with such activity into the blood following chemotherapy. The frequency of lymphocytes expressing FcR, which is a trait of most NK-cells (for review see 15) was found to be increased, but not to a significant extent. It could be postulated that the differences observed were not due to the treatment but to the disease per se. This is unlikely, however, since breast carcinoma patients have been shown to have a reduced NK activity compared with women with benign breast disease (9).

An attempt to split the patient material into 2 groups depending on the type of chemotherapy they had received did not reveal any significant differences with respect to any parameter studied (data not presented).

Taken together, our previous and present results indicate that cyclic LMF or CMF therapy for breast carcinoma induces relatively mild changes of the blood lymphocyte population. Lymphocyte stimulations, which are somewhat depressed during the acute phase, are normalized within 4 to 6 years. Although of low-grade statistical significance ($p<0.05$) our results indicate that the chemotherapy induced increment of NK activity persists for several years. A larger patient material, a larger panel of target cells and a longer follow-up period of the patients are required to study this phenomenon in more detail. Whether the induction of an increased NK activity is one mechanism by which LMF or CMF therapy exert their antitumor activity is unknown.

ACKNOWLEDGEMENTS

The authors wish to thank Mrs Cecilia de Laval for her excellent technical assistance and Dr Bo Nilsson for his expert advice concerning statistical evaluation of the results. This work was supported by grants from King Gustaf the Vth Jubilee Foundation.

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