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ADJUVANT RADIATION THERAPY COMPARED WITH CYCLIC CHEMOTHERAPY IN PATIENTS WITH MAMMARY CARCINOMA

II. Changes of mitogen responses of blood lymphocytes

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The treatment of choice for most patients with breast carcinoma is surgery. However, there is a considerable risk of recurrence, particularly in patients with a relatively advanced disease. Adjuvant radiation therapy or chemotherapy may increase survival (BONADONNA & VALAGUSSA 1981, WALLGREN et coll. 1980). However, it is not yet established if these adjuvant treatments differ in preventing tumor recurrences. A controlled clinical trial is in progress in the Stockholm area to compare the clinical value of postoperative local radiation therapy with cyclic chemotherapy.

Previously, PETRINI et coll. (1981) compared the effects of adjuvant radiation therapy and cyclic chemotherapy on the size of various blood lymphocyte subsets as defined by membrane markers. In the present article the effects of local postoperative radiation therapy are compared with cyclic chemotherapy (chlorambucil, methotrexate and 5-fluorouracil) on the functional activity of the blood lymphocytes as measured by mitogenic responses to purified protein derivative of tuberculin (PPD) and phytohaemagglutinin (PHA) in vitro.

Material and Methods

The series consisted of 62 patients with operable carcinoma of the breast. All the patients underwent a modified radical mastectomy including extirpation of the axillary lymph nodes en bloc. Postoperative adjuvant chemotherapy was administered to 44 patients, 25 to 70 years old (mean 54) and postoperative radiation therapy to 18 patients, 33 to 70 years old (mean 56). The patients were included in a clinical trial aiming at comparing the value of adjuvant chemotherapy with adjuvant radiation therapy in patients with primary tumors exceeding 3 cm in diameter or in patients with histologically confirmed malignant involvement of axillary lymph nodes, irrespective of the size of the tumor. The postmenopausal patients included in the trial were also randomized to adjuvant anti-oestrogen treatment with tamoxifen (Nolvadex ICI), 40 mg/day orally, or no anti-oestrogen treatment. This treatment was started together with adjuvant chemotherapy and continued for 2 years. Fifteen of the patients who received adjuvant chemotherapy and 5 who received radiation therapy also received tamoxifen.

Chemotherapy was started 4 to 6 weeks after surgery. The patients received 600 mg/m^2 of 5-fluorouracil and 50 mg of methotrexate intravenously on day 1 and 8 during each cycle and chlorambucil, 15 mg daily per os, day 1 through 8. The next cycle started on day 42. The treatment included 12

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such cycles and consequently it took around 17 months to complete the whole treatment. The doses were reduced in women 65 years of age or older or according to hematologic toxicity or other side effects. In most patients the doses were reduced in one or more cycles. Table 1 shows the range and mean of the total doses in per cent of the initially planned total doses. Two patients received cyclophosphamide instead of chlorambucil in the last 4 and 7 cycles, respectively, because of thrombocytopenia. In 7 patients the last 4 cycles were given at an interval of 3 months. No correction has been made for these deviations from the treatment protocol when calculating lymphocyte reactivity to PPD and PHA, since the values were similar to those of the rest of the patients.

Radiation therapy. Four to six weeks after surgery the operated area of the chest wall was irradiated using a 6 to 8 MeV electron beam. The internal mammary and supraclavicular regions on the homolateral side were irradiated using an anterior ^{60}Co portal and the target dose was calculated at a depth of 3 cm. The axillary region was irradiated by an anterior and posterior ^{60}Co field and the dose was calculated in the mid-axilla. A dose of 46 Gy was delivered to all regions in about 5 weeks in 23 fractions. One patient received only 38 Gy because of a more profound skin reaction in the irradiated area.

Blood sampling. Sample I was obtained immediately before the adjuvant treatment. From the patients who received adjuvant chemotherapy sample II was obtained on day 8, No. III on day 22, and No. IV on day 42, i.e. immediately before the second cycle. Samples No. V through IX were obtained immediately before the fourth, sixth, eighth, tenth and twelfth cycle. Sample X through XIII were obtained approximately 3 (±2 weeks), 9 (±4 weeks), 15 (±8 weeks), and 21 months (±10 weeks), after the last cycle of chemotherapy. From the patients who received adjuvant radiation therapy, sample II was drawn after completion of the treatment and sample III 3 weeks (±1 week) later followed by sample IV through IX obtained approximately 3 (±2 weeks), 6 (±2 weeks), 9 (±3 weeks), 15 (±2 weeks), 21 (±5 weeks) and 27 (±4 weeks) months after the treatment. For trivial reasons all samples were not obtained from all patients. Data obtained within 3 months of detection of recurrent disease are not presented.

Separation of lymphoid cells. Lymphoid cells were separated from heparinized venous blood by centrifugation on a layer of Ficoll-Isopaque (BÖYUM 1968). The cells were then washed twice by centrifugation in Eagle's Minimal Essential Medium supplemented with Earle's salts (MEM). These cell preparations contained approximately 90 to 95 per cent of small lymphocytes.

Lymphocyte stimulants. Two different stimulants were used to induce DNA synthesis in lymphocytes. (1) Purified protein derivative of tuberculin (PPD tuberculin, RT 23, Statens Seruminstitut, Copenhagen, Denmark). The lymphocytes were cultured with PPD at final concentrations of 0.1 and 1.0 μg/ml. The same batch of PPD was used in all patients. (2) Phytohaemagglutinin (PHA, Bacto-phytohaemagglutinin M., Difco Lab. Detroit, Mi. USA).

Table 1

Total doses of the 3 cytostatic drugs given to the patients. The doses are expressed in per cent of the total doses which were originally planned

Drug	Range (per cent)	Mean (per cent)
Chlorambucil*	56–100	89
Methotrexate	39–100	79
5-fluorouracil	45–104	78

* Two patients who received cyclophosphamide instead of chlorambucil are excluded.

Table 2

Stimulatory activity of 3 different batches of PHA for lymphocytes obtained from patients before start of adjuvant therapy (sample I). Values are expressed as mean log_{10} cpm ± SE. The stimulatory activity of batch I differed significantly from batch II and III ($p<0.001$). No significant difference existed between batch II and III. Values within parentheses show the antilogarithmized mean values (geometric mean)

	No.	
Batch I	44	5.07±0.04 (117 100)
Batch II	31	4.85±0.05 (70 800)
Batch III	27	4.76±0.04 (57 360)
Batch II+III	58	4.81±0.03 (64 200)

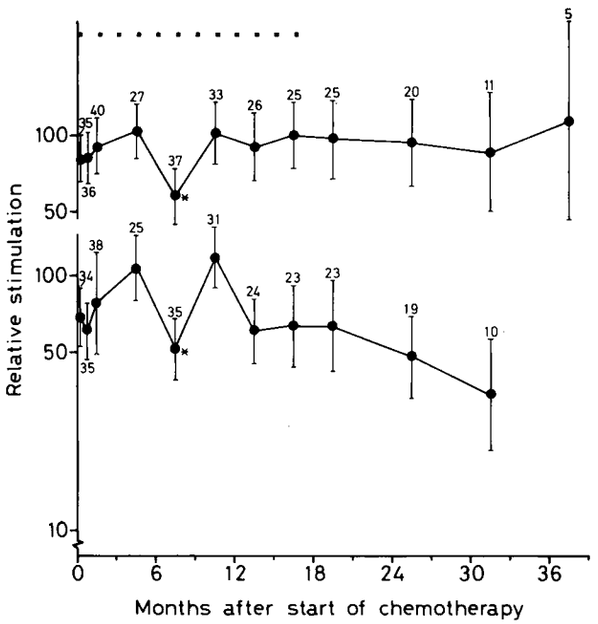

Fig. 1. Relative changes (in per cent) of PPD stimulation of the blood lymphocytes during and after adjuvant chemotherapy. Two different PPD concentrations were used 1.0 μg/ml (top curve) and 0.1 μg/ml (bottom curve). Mean values and SE are calculated. Figures at the symbols show the number of patients tested. The degree of statistical significance is indicated with *(<0.05). Each cycle of chemotherapy is indicated at the top (■).

Table 3

Initial values (mean ± SE) for each stimulant in the 2 different treatment groups expressed as $\log_{10}$ cpm. No statistically significant differences were found. Differences between means were statistically non-significant

Stimulant	Post-operative radiation therapy	No. of tests	Post-operative chemo-therapy	No. of tests
PPD 1.0 μg/ml	3.99±0.13	17	3.93±0.09	44
PPD 0.1 μg/ml	3.55±0.16	18	3.51±0.12	42
PHA	5.05±0.05	16	5.05±0.02	43

The contents of vials of PHA were dissolved in 5 ml of MEM (100% solution). The lymphocytes were cultured with PHA at a final concentration of 3 per cent (v/v). Three different batches were used consecutively.

Lymphocyte stimulation tests. A microculture technique was used which has been described in detail previously (LILLIEHÖÖK & BLOMGREN 1974). Briefly, lymphoid cells were cultured in the wells of microtest plates containing 0.2 ml of MEM, 10 per cent of heat inactivated human serum and antibiotics. Each culture contained 10^5 lymphocytes. Control cultures, without mitogens, contained the same number of cells. All cultures were set up in triplicate. They were incubated at 37°C in a humidified 5% CO_2-atmosphere. Twenty-four hours before termination each culture received 1.0 μCi of ^{3}H-thymidine (185 GBq (5 Ci)/mmol, The Radiochemical Centre, Amersham, England). PPD stimulated cultures were terminated on day 7 and PHA stimulated on day 5.

Incorporated radiation activity, expressed as counts per minute (cpm), was determined as described previously (LILLIEHÖÖK & BLOMGREN). Activity of the control cultures was subtracted from the values of the corresponding experimental cultures.

Statistical methods and data processing. The initial value (sample I) of each type of determination for each patient was set at 100 per cent. The values of the subsequent tests were related to this value. The lymphocyte stimulations, after having subtracted the values of the control cultures, were close to zero or even below zero in some tests. In these tests the stimulations were arbitrarily given a value of 5 per cent.

Mean values and standard errors (SE) were calculated on a geometric basis. Statistical probability values were determined using the Student's t-test.

Three different batches of PHA were used: the first from the beginning of the project until February 1978, the second from February 1978 until January 1979 and the third from January 1979. It appeared that these batches differed in their capacity to stimulate DNA synthesis of lymphocytes. The mean stimulations of lymphocytes obtained from sample No. I using the three PHA batches appear in Table 2.

The first batch was significantly more stimulatory ($p<0.001$) than the second and the third. No difference existed between the second and the third batches. Since the mitogen responses of a patient's lymphocytes are related to the initial value (sample I) correction was made when the initial value was obtained using the first batch and the subsequent tests with the second or the third. The correction factor of 1.82 was the quotient betweeen the mean value of sample I using the first batch and the mean value of sample I using the second and the third batches (antilogarithmized mean values). Thus, the absolute value of a stimulation test, expressed as

cpm, was multiplied by the correcting factor and thereafter the background isotope uptake of the control culture was subtracted. The same PPD-batch was used throughout the series. The initial PPD-stimulation values obtained during the three time periods when various PHA-batches were used, did not differ significantly (data not shown).

Results

In Table 3 the initial PPD and PHA stimulation values expressed as $\log_{10}$ of cpm, are shown for patients given chemotherapy or radiation therapy. No statistically significant differences were noted between the groups.

PPD stimulations

Chemotherapy. Two patients, being anergic to PPD at a concentration of 0.1 μg/ml before treatment (sample I), were excluded. The PPD responses varied extensively during the treatment (Fig. 1). Approximately 8 months after start of treatment, a significant decrease in lymphocyte stimulation occurred using both concentrations of PPD ($p<0.05$). This was the only significant change of PPD reactivity during and after treatment.

Radiation therapy. One patient being anergic to PPD at a concentration of 1.0 μg/ml before treatment, was excluded. Immediately after irradiation a significant decrease in lymphocyte stimulation for both concentrations of PPD ($p<0.001$) occurred. Subsequently PPD reactivity gradually increased and about 6 months after irradiation it reached a plateau at approximately 40 per cent of the initial value for the higher and approximately 30 per cent for the lower concentrations of PPD (Fig. 2).

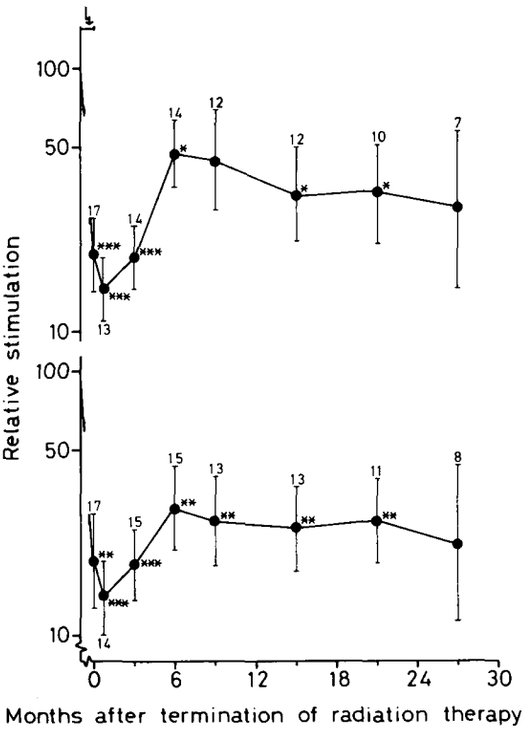

Fig. 2. Relative changes (in per cent) of PPD stimulations of blood lymphocytes after adjuvant radiation therapy. Two different PPD concentrations were used, 1.0 μg/ml (top curve) and 0.1 μg/ml (bottom curve). The period of irradiation is indicated with an arrow. The degree of statistical significance is indicated with * (<0.05), **(<0.01) and ***(<0.001).

PHA stimulations

Chemotherapy. In one patient the initial value was missing and she was thus excluded. The PHA reactivity of the lymphocytes decreased continuously during the treatment (Fig. 3). The reduction became significant after the ninth cycle of chemotherapy and continued for 9 months after the last cycle when it reached approximately 45 per cent of the initial value. Thereafter a rise in PHA stimulation occurred and about 21 months after the last cycle of chemotherapy it seemed to reach the initial value.

Radiation therapy. The initial value was missing in 2 patients who were thus excluded. At completion of the radiation therapy a significant decrease of reactivity to approximately 45 per cent of initial values was noted ($p<0.001$). A partial restoration followed 3 to 6 months later (Fig. 4). Thereafter PHA reactivity decreased again and about 21 months after radiation therapy it reached a plateau at approximately 40 per cent.

Discussion

The aim of postoperative adjuvant chemotherapy or radiation therapy in mammary carcinoma is to decrease the recurrence rate and increase survival (BONADONNA & VALAGUSSA, WALLGREN et coll.). Both treatment modalities have side effects which are important to map. In the present series the effects of these adjuvant treatments on the mitogen reactivity of blood lymphocytes were compared. The effects of local radiation therapy on the blood lymphocyte population is reasonably well known (BLOMGREN et coll. 1974, 1977, BARAL et coll. 1977. PETRINI et coll. 1977, CAMPBELL et coll. 1976,

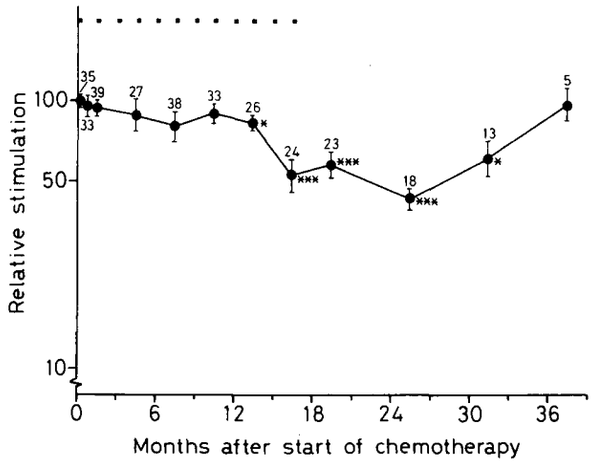

Fig. 3. Relative changes (in per cent) of PHA stimulation of blood lymphocytes during and after adjuvant chemotherapy.

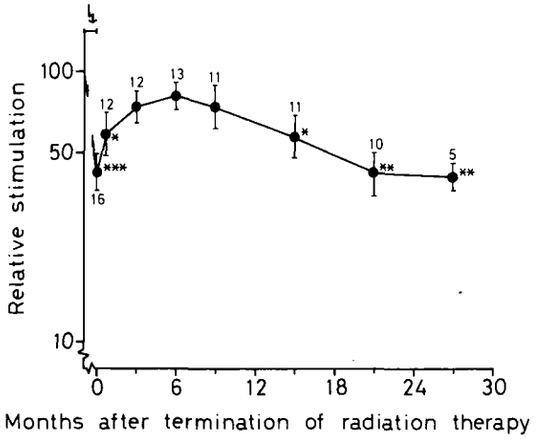

Fig. 4. Relative changes (in per cent) of PHA stimulation of blood lymphocytes after adjuvant radiation therapy.

CHEE et coll. 1974) whereas still only few reports on the effects of postoperative chemotherapy in breast carcinoma have appeared (PETRINI et coll. 1979, 1981, STRENDER et coll. 1981).

In the present series those patients who were randomized to radiation therapy serve as a control group to those who received chemotherapy. The 15 postmenopausal patients who received tamoxifen in addition to chemotherapy did not differ significantly in lymphocyte reactivity to PPD and PHA compared with those 13 postmenopausal patients who did not receive tamoxifen (data not presented). The same comparison could not be made for patients who were given radiation therapy since only 5 patients received tamoxifen. During and after cyclic chemotherapy no significant change in the PPD-reactivity of the lymphocytes occurred except for sample number VI where a decrease of low grade statistical significance was observed (Fig. 1). Notwithstanding the great variability of the determinations there seemed to be a decrease of the response to the lowest PPD concentration employed (0.1 μg/ml). This reduction was most apparent at the end of the chemotherapy and became more prominent after completion of the treatment. If confirmed these results indicate that PPD-memory T-cells are relatively resistant to this type of chemotherapy and an injury to these cells can only be detected when a highly sensitive assay is used, i.e. when the lymphocytes are exposed to suboptimally stimulatory concentrations of the antigen. In contrast to chemotherapy irradiation sharply reduced the response of the lymphocytes to both concentrations of PPD. The reduction was most marked at completion of irradiation followed by a rapid partial recovery during the first 6 months after treatment. These findings are essentially in agreement with previous results (BLOMGREN et coll. 1976, BARAL et coll.).

The lymphocyte reactivity to PHA seemed to decrease gradually during chemotherapy and continued to do so for 9 months after its completion followed by a recovery. These results are in agreement with other reports showing reductions of PHA reactivity during chemotherapy with various drugs in patients with advanced malignant tumors (HERSH & OPPENHEIM 1967, CARDOZO 1970, AL SARRAF et coll. 1972, ROMAGNANI et coll. 1976). There may be several explanations for the reduced PHA response in patients treated with chemotherapy. PHA reactive T-cells may have been relatively selectively killed by the treatment or may have been sublethally injured in such a way that they cannot undergo adequate cell division. Another possibility is that the reduced response was due to a relative deficiency of non-T-lymphocytes during and after this type of chemotherapy (PETRINI et coll. 1981). Such cells are known to augment the PHA response of T-cells (BLOMGREN 1976). Immediately after irradiation the PHA response was decreased but later followed by a transient restitution and a second decrease (Fig. 4). In some of the previous reports no reduction of the relative PHA response after radiation therapy was observed (BARAL et coll., BLOMGREN et coll. 1974, 1976). This discrepancy can possibly be attributed to different culture techniques employed (IDESTRÖM et coll. 1979) and it may be concluded that the present culture technique is more sensitive and may in a more adequate way reveal the changes

of the lymphocyte populations to respond to mitogenic stimuli.

In conclusion, the results of this investigation have shown that both postoperative radiation therapy and adjuvant cyclic therapy with chlorambucil, methotrexate and 5-fluorouracil impair the immunologic reactivity of blood lymphocytes as measured by PHA and PPD stimulations in vitro. The radiation therapy used seems to cause more severe suppression of the PPD response than the chemotherapy regime used in the present series both with respect to its extent and duration.

SUMMARY

Blood lymphocyte reactivity to purified protein derivative of tuberculin (PPD) and phytohaemagglutinin (PHA) was examined in 62 patients with breast carcinoma who received postoperative adjuvant radiation therapy or cyclic chemotherapy with chlorambucil, methotrexate and 5-fluorouracil. Both treatments impaired the immunologic reactivity of blood lymphocytes as measured by PPD and PHA stimulation in vitro. Radiation therapy seemed to cause more profound and protracted suppression of the PPD response than chemotherapy.

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