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Adjuvant treatment of breast cancer: A pilot immunochemotherapy study with CMF, interleukin-2 and interferon alpha

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Abstract Immune responses, including natural immunity (NI), potentiate the antitumor effects of chemotherapy. Since interferons and interleukin-2 (IL-2) augment NI, a pilot study was conducted to assess the tolerability and the effects on host immunity of adjuvant chemotherapy associated with IL-2 + interferon alpha (IFN) in breast cancer patients after surgery. Ten patients underwent alternating 28-day cycles of chemoimmunotherapy [cyclophosphamide + methotrexate + 5-fluorouracil (CMF, days 1, 8) + IL-2 (days 15–19) + IFN (day 22)] and chemotherapy alone (CMF). With this regimen each patient was considered to be a reasonable "control" of herself. Blood cell count and natural killer cell activity (NKA) were tested on days 1, 8, 15, 22, and 23. Preliminary in vitro studies indicated that IL-2 or IFN antagonized the severe inhibition of NKA induced by hydroxy-peroxy-cyclophosphamide (in vitro active derivative of cyclophosphamide), alone or associated with methotrexate + 5-fluorouracil. Nine patients completed all six alternating cycles, whereas one patient proved to have metastatic lesions after four cycles. The protocol was well tolerated, although leukopenia (CMF alone) and leukopenia with fever and moderate or minimal flu-like symptoms (CMF + IL-2 + IFN) were generally observed. Treatment with IL-2 facilitated complete recovery of white cell counts and NKA after the nadir on day 15. In conclusion, the present protocol appears to be well tolerated and amenable to administration on an outpatient basis. Therefore, further investigations should be performed to verify whether CMF + IL-2 + IFN would be superior to CMF alone for adjuvant treatment after surgery in breast cancer.

Key words Interferon alpha · CMF · Breast cancer · Chemo-immunotherapy

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Introduction

Previous clinical studies suggest that impaired immune function is associated with a poor prognosis in breast cancer [1, 6, 15]. Moreover, local treatment (i.e. surgery and/or radiotherapy) or systemic chemotherapy may cause some degree of immunodepression [27, 40, 50, 54, 56]. Therefore, it is reasonable to hypothesize that minimizing immunosuppression could favorably influence the course of the disease [27]. For this reason, immunostimulating agents have been studied in the treatment of various types of cancer, including breast cancer [27, 34, 52].

Preclinical investigations have shown that the therapeutic effects of antineoplastic agents can be potentiated by increasing or sustaining the host's immune responses against immunogenic tumors [7, 39, 41]. Moreover, it was found that significant amplification of the antitumor effect of anticancer drugs can be supported by natural immunity (NI) in vivo [31].

Interferons, especially alpha interferon, have shown antitumor effects in animal systems [8, 20–22] and in cancer patients [5, 24, 30, 37]. Interferons also substantially increase various types of immunity in animals and man, such as natural killer (NK) cell-mediated cytotoxicity [42] and antibody-dependent cellular cytotoxicity [28]. Therefore, it appears reasonable to postulate that the therapeutic effects of interferons could be mediated, at least in part, by amplification of the antitumor

effects of antineoplastic agents on the basis of immunochemotherapeutic synergism [7, 39, 41].

Interleukin-2 (IL-2), produced mainly by CD4+ T-cells, has a wide variety of activities and has a central role in the regulation of immune system [51]. The primary function of IL-2 is to promote the growth of activated T-cells that bear IL-2 receptors [49]. However, IL-2 has an array of other effects on B-cells [57], macrophages [25, 36], epidermal Langerhans cells [48] and oligodendroglial cells [4]. Many of the biological effects of this cytokine suggest that it might be of value in cancer therapy [44]. The molecule causes lymphoid cell proliferation (i.e. progression from phase Gl to phase S of the cell cycle), and reverses down-regulation of a number of NK and T-cell functions both in vitro and in vivo [43, 51]. Administration of IL-2 restores depressed allogeneic responses in cyclophosphamide-treated mice [38]. The lymphokine causes proliferation of both endogenous and adoptive transferred lymphoid cells in vivo [16, 17] and generates lymphokine-activated killer cells (LAK) [44], which are capable of lysing NK-resistant fresh tumor cells.

Since both interferons and IL-2 may be able to increase a host's immune reactivity in association with chemotherapy, including NI function, it is conceivable that sequential treatment with IL-2 and interferon would result in additive or synergistic immunoenhancing effects. Moreover, since interferons are often endowed with direct cytostatic activity on tumor cells, it is possible that an IL-2 + interferon combination would be particularly active in ameliorating the efficacy of anticancer drugs. Therefore, a pilot study has been designed to test the feasibility of an adjuvant chemoimmunotherapeutic approach in breast cancer patients using cyclophosphamide + methotrexate + 5-fluorouracil (CMF) combined with IL-2 + interferon. In particular, the present investigation was performed to establish whether low immunomodulating doses of IL-2 + interferon would influence the hematological and immunological toxic effects of chemotherapy.

The results of the present study show that this new approach is well tolerated. Moreover, treatment with IL-2 + interferon after chemotherapy was followed by accelerated recovery of the NK function and of the number of lymphocytes, monocytes and eosinophils in peripheral blood.

Materials and methods

Drugs and reagents

Recombinant IL-2, lyophilized vials containing 18×10^6 IU/vial (Roche, Milan, Italy), recombinant interferon-alpha-2a (IFN, lyophilized vials containing 10^6 IU/vial, Roche), 5-fluorouracil (5-FU, 250 mg/vial, Roche). methotrexate (MTX, 50 mg/vial, Lederle Cyanamid Laboratories, Catania, Italy) and cyclophosphamide (CY, 500 mg/vial; Endoxan, Asta Medica, Milan, Italy) were commercially available; 4-hydroxy-peroxy-cyclo-phosphamide (HPC), an in vitro-active derivative of CY, and MTX for in vitro studies were kindly supplied by Schering (Milan, Italy) and Lederle Cyanamid Laboratories, respectively.

Tumor cells

The human cell line K562 [35] was maintained in tissue culture in RPMI-1640 medium (Flow Laboratories, McLean, Va.) supplemented with 10% fetal calf serum (FCS, Flow), 2 mM gultamine (Flow) and 50 μg/ml gentamicin (Flow) (hereafter referred to as "complete medium," CM).

Preparation of mononuclear cells and drug treatment in vitro

Peripheral blood mononuclear cells (MNC) were separated from heparinized whole blood on a Ficoll-Hypaque gradient, washed twice in RPMI-1640 medium containing 2% FCS, 10 mM Hepes (Flow), and 50 μg/ml gentamicin (hereafter referred to as "washing medium," WM) and resuspended in CM at the desired concentration.

Freshly prepared MNC (10^6 cells/ml) obtained from healthy donors were incubated with HPC (10 μg/ml), MTX (4 μg/ml), or 5-FU (60 μg/ml), or with HPC + MTX + 5-FU (HMF, i.e. the in vitro-active CMF association, 10 μg/ml, 4 μg/ml, and 600 μg/ml, respectively) in 24-well tissue culture plates (Becton Dickinson, Oxnard Calif.) in a total volume of 2 ml/sample at 37 °C for 1 h.

Additional groups were exposed to IFN (100 IU/ml for 2 h at 37 °C, 1 h before treatment with antitumor drugs), or to IL-2 (500 IU/ml, for 4 days, beginning after the end of treatment with the antineoplastic agents). The concentration of HPC was selected on the basis of the results obtained by Kodo et al. [33], showing that profound inhibition of NK activity (NKA) is obtained by treating MNC in vitro with 9–12 μg/ml of HPC. The concentrations of MTX and 5-FU were selected using the same MTX/5-FU ratio as was adopted for the three drug combination schedule in vivo according to previous studies performed in our laboratory [52]. Moreover, the concentration of HPC was maintained at 10 μg/ml, as previously described [52]. Actually higher concentrations of this agent were found to be drastically suppressive of NKA, and not usable in drug combination experiments (data not shown). At the end of the drug treatment MNC were washed twice and resuspended at the desired concentration, after which they were used as effector cells against ^{51}Cr-labeled K562 target cells.

Labeling of target cells for cytotoxicity assay

K562 target cells were suspended in 0.1 ml of FCS, labeled with 100 μCi of $^{51}Na_2CrO_4$ (Amersham International, Amersham, Bucks., UK), and incubated in 5% CO_2 humidified atmosphere at 37 °C for 1 h. After incubation, the cells were extensively washed with WM and resuspended in CM at the desired concentration.

Cytotoxicity assay

Effector MNC in 0.1 ml of CM were plated in quadruplicate in U-bottomed 96-well microtiter plates (Greiner, Nurtingen, Germany) by making serial two-fold dilutions starting at the concentration of 2×10^6 cell/ml. Washed labeled target cells (2×10^4) were added in a volume of 0.1 ml to give effector-to-target cell ratios ranging from 100:1 to 12.5:1. The plates were then incubated at 37 °C in a 5% CO_2 humidified atmosphere for 4 h. At the end of incubation the plates were centrifuged at 250 g for 10 min and 0.1 ml of supernatant was collected and counted in a gamma scintillation counter (5142, Packard Instruments, Downers Grove, Ill.).

The means of counts per minute (cpm, quadruplicate samples) were expressed as the percentage of specific lysis according to the formula:

$$\% \text{ Specific lysis} = \frac{\text{cpm (test)} - \text{cpm (autologous control)}}{\text{cpm (total count)}} \times 100$$

Where cpm (test) is the mean radioactivity released in the presence of effector cells, cpm (autologous control) is the mean radioactivity released by target cells incubated with 2×10^5 unlabeled autologous cells in place of effector cells, and cpm (total count) is the total amount of ^{51}Cr incorporated into target cells.

Evaluation of cytotoxic activity of effector cells in terms of killed cells

The cytotoxic activity of effector cells was calculated using a data processing technique developed in our laboratory [53]. All values were expressed in terms of number of target cells lysed (i.e., "killed", killed cells or KC) by a fixed number of effector cells, as follows:

$$KC(m) = \frac{m \times T \times n\%}{En \times 100}$$

Where KC(m) is the number of target cells killed by m effector cells, T is the total number of target cells present in each well, En is the number of effector cells present in each well at the effector:target cell ratio selected for calculation, and n% is the specific lysis produced theoretically by En effector cells. The n% value is extrapolated from the best-fit curve obtained by plotting the different percentages of specific lysis vs ln of the number of effector cells/well, at graded effector:target cell ratios. In the present study KC(m) was calculated for $m = 10^6$ cells [i.e. $KC(10^6)$], and $En = 2 \times 10^6$ cells (i.e., at effector:target cell ratio of 100:1).

When MNC of cancer patients were tested for NKA, KC was also calculated in terms of KC (ml), i.e. for m = number of lymphocytes present in 1 ml of blood.

Statistical analysis

Statistical evaluation of all measured parameters in cancer patients was performed as follows. All mean values for hematological, biochemical and immunological parameters were calculated from pooled data collected from all patients for all cycles together on day l. For the other days of the tests, all mean values relative to hematological and biochemical parameters were calculated from pooled data collected from all patients for all cycles of chemo-immunotherapy (i.e. all cycles "A", see below), or of chemotherapy only (i.e. all cycles "B", see below) separately. However, l patient developed lung metastases during the fifth cycle of therapy (i.e. in this case during the third cycle of chemo-immunotherapy). For this patient, therefore, four cycles only were considered for statistical analysis. All mean values for immunological parameters were calculated from pooled data collected from all patients for the first three cycles of therapy, except for the results illustrated in Fig. 4B. In all cases, mean differences were statistically evaluated according to non-paired or paired Student's *t*-test analysis.

In the in vitro studies the paired Student's *t*-test was also utilized to compare data within the same experiment.

Immunofluorescence staining and cytofluorimetric analysis

Patients' MNC were washed twice in phosphate-buffered saline (PBS) supplemented with 0.1% bovine serum albumin and 0.02% sodium azide (Sigma; (PBS-A), and 1×10^6 cells were then suspended in 50 µl of PBS-A containing the appropriate monoclonal antibody (mAb). For the negative control, MNC were incubated with fluorescein isothiocyanate (FITC)-conjugated mouse I_gG_1 and phycoerythrin (PE)-conjugated mouse I_gG_1. Samples were incubated at 4 °C for 30 min and then washed twice in PBS-A. The labeled samples were analyzed with a FACScan (Becton Dickinson FACS system). Data were collected on 1×10^4 viable cells as determined by forward and side-angle light scatter. Data analysis was performed by using "Consort 32" software on Hewelett Packard computer (Hewelett Packard, Fort Collins, USA). The following mAbs were utilized for two-color analysis: FITC-conjugated anti-CD4 (IgG_1, SK_3 hybridoma), anti-CD16 (IgG_1, NKP15 hybridoma) and anti-CD25 (IgG_1, 2A3 hybridoma), PE-conjugated anti-CD8 (IgG_1, SK1 hybridoma). All control immunoglobulins and mAbs were purchased from Becton Dickinson.

Patients

Ten patients with histologically confirmed breast carcinoma in stages I and II (grading G1–G3) and 29–67 years old (4 premenopausal, 6 postmenopausal) entered the study after surgery. Informed consent was obtained from all patients. The ethical committee of our University also approved the protocol. Two patients (stage I, T1cN0) were treated with wide resection + axillary dissection followed by postoperative irradiation therapy to the axilla with at least 50 Gy. The remaining patients, (one in stage I: T1cN0 and seven in stage II: T2NO-1, T3NO) underwent modified radical mastectomy with axillary dissection. Six tumors were ER+ and 5 were PR+. Inclusion criteria were: (1) no bilateral or previous breast cancer; (2) no prior biological systemic therapy; (3) no prior systemic chemotherapy or endocrine therapy; (4) no steroid therapy; (5) age less than 75 years; (6) number of positive lymph nodes no more than 3; (7) indifferent menopausal status; (8) Karnofsky performance status greater than 60; (9) adequate bone marrow reserve, as defined by peripheral white cell count not less than 4000 cells/µl, hematocrit not less than 30% and platelet count not less than 100,000 cells/µl; (10) adequate hepatic and renal functions; (11) no nonmalignant systemic disease that would preclude therapy or prolonged follow-up; (12) no psychiatric or addictive disorder that would preclude informed consent; (13) no prior or concurrent malignancy except for nonmelanomatous skin cancer, and adequately treated in situ carcinoma of the cervix; (14) no pregnancy or breast feeding; (15) no significant cardiovascular disease within 2 years, including myocardial infarction, arrhythmia, cardiac heart failure, coagulation disorders (i.e. thrombophlebitis); (16) no symptomatic pulmonary disease, PO_2 at least 60 mm Hg, no history of pulmonary embolism; (17) adequate contraception (required for fertile women); (18) geographical accessibility for frequent follow-up observation.

Clinical study design

The study design adopted in the present investigation is based on alternating cycles of chemotherapy and of chemo-immunotherapy, as previously described [13, 14]. Treatment schedules are illustrated in detail in Fig. 1. All patients underwent alternating cycles of chemo-immunotherapy (i.e. cycle "A," CMF + IL-2 + IFN) and chemotherapy alone (i.e. cycle "B," CMF), for a total of six cycles. Medical treatment started after complete recovery from prior surgery, or at least 3 weeks after radiotherapy.

Treatment schedule of chemo-immunotherapy was designed according to the following sequence: (a) step 1: standard chemotherapy with CY + MTX + 5-FU (days 1, 8); (b) step 2: recovery of NI function, possibly depressed by chemotherapy, using daily treatment with immunomodulating doses of IL-2 (days 15–19) and IFN (day 22). Alternate patients were assigned to A and B as the initial cycle. The first patient started with cycle A. The last patient was included in the list of patients entering on cycle A instead of B. In fact, one patient who started with cycle A developed lung metastases during the fifth cycle; this patient was withdrawn from the study and replaced with another patient, who was scheduled to start with cycle A. Therefore, a total of six patients started with cycle A and four, with cycle B. Alternating cycles (i.e., cycle A and cycle B alternately) were administered to all patients, so that each patient could be analyzed as a reasonable control for herself [13, 14].

Treatment schedule and doses

All patients were treated with CY (600 mg/m^2, i.v., bolus, days 1, 8 of each cycle) + MTX (40 mg/m^2, i.v., bolus, days 1, 8 of each cycle) + 5-FU (600 mg/m^2, i.v., bolus, days 1, 8 of each cycle). During chemo-immunotherapy cycles all patients also received IL-

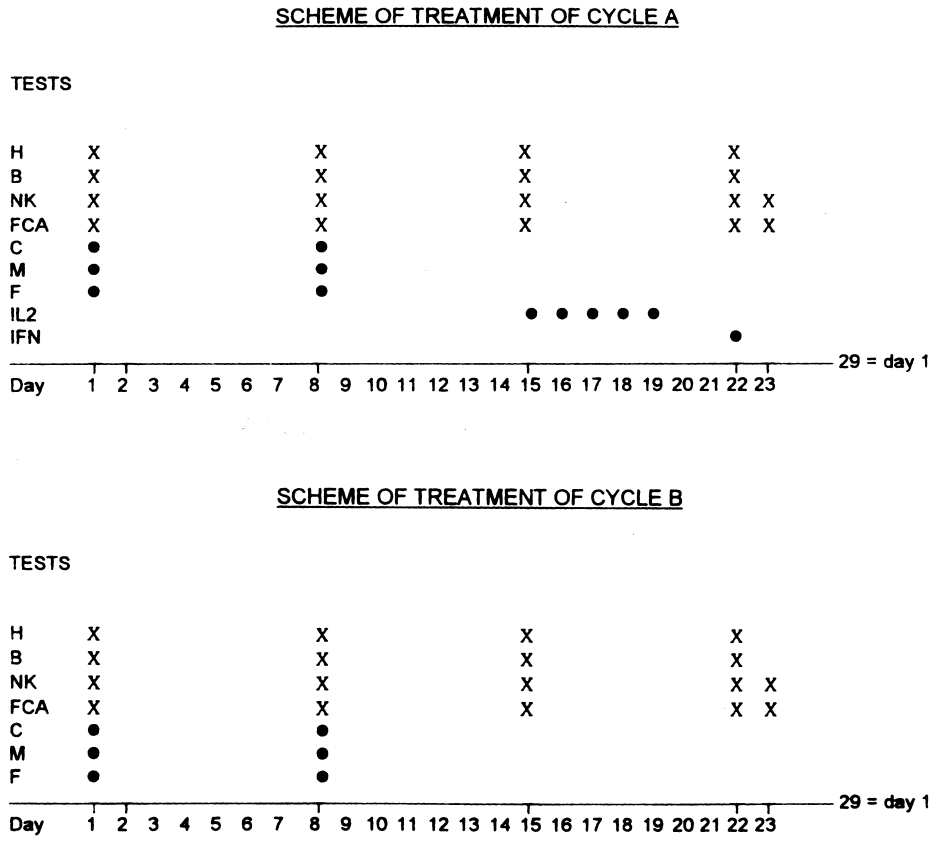

Fig. 1 Treatment schedule of breast cancer patients subjected to chemoimmunotherapy [cycle A: cyclophosphamide + methotrexate + 5-fluorouracil (CMF) + recombinant α-2a (IFN): + interleukin-2 (IL-2)] or to chemotherapy alone (cycle B: CMF)in alternating cycles for a total of six cycles. Each cycle was repeated every 29 days [*C* cyclophosphamide (600 mg/m^2 i.v., days 1, 8), *M* methotrexate (40 mg/m^2 i.v., days 1,8), *F*-5-fluorouracil (600 mg/m^2 i.v., days 1, 8); IL-2 (3 MIU/m^2 s.c., days 15–19); alpha-IFN (3 MIU/m^2 i.m., day 22, *H* hematology (i.e. erythrocytes, hemoglobin, WBC, and platelet counts), *B* blood chemistries (i.e. electrolytes, LDH, SGOT, SGPT, gamma-GT, bilirubin, alkaline phosphatase, BUN, creatinine, red cell sedimentation), and urinalysis, *NK* assay for natural killer (NK) cell activity of mononuclear cells (MNC), *FCA* flow cytometric analysis of CD16 + CD25 + CD4 + and CD8 + lymphocyte subpopulations

2 (3 MUI/m^2, sc., daily for 5 days: days 15–19) and IFN (3 MUI/m^2, day 22). All cycles were 4-weeks long.

Toxicological, immunological and clinical monitoring

The number of blood cells (i.e. erythrocytes, granulocytes, lymphocytes and platelets) microliter and the blood concentration of hemoglobin (g/100 ml) were tested on days 1, 8, 15 (i.e. before IL-2 administration), and on day 22 (i.e., after IL-2 and before IFN administration) of each cycle. Evaluation of NI (i.e. NKA determination) and FACS analysis for lymphocyte subpopulations were performed on days 1, 8, 15, and 22, and additionally on day 23 (i.e. after IFN administration).

Electrolytes (i.e. Na^+, K^+, Ca^{2+}, and Mg^{2+}), LDH, SGOT, SGPT, gamma GT, bilirubin, alkaline phosphatase, BUN, and creatinine were determined on day 1 of each cycle, along with urinalysis.

Evaluation of the toxicological and clinical course of the disease was completed by physical examination on day of each cycle.

At the end of this treatment, nine patients entered follow-up, consisting in physical examination, hematology, blood chemistries and urinalysis every 3 months; chest-X-ray and echography of the upper abdomen and tumor markers every 6 months; and mammography and bone scan every 12 months. At present the patients have reached a follow-up time of 36–40 months. One patient developed lung metastases during the fifth cycle and embarked on a protocol for advanced disease.

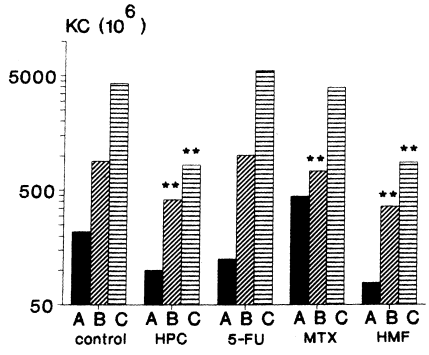

Fig. 2 In vitro effects of 1 h treatment with 4-hydroxy-peroxy-cyclophosphamide (*HPC*, 10 μg/ml), 5-FU (60 μg/ml), or MTX (4 μg/ml), or with HPC + 5-FU + MTX (*HMF*) alone or in combination with IFN (10 IU/ml for 2 h, before exposure to the drugs), or with IL-2 (500 IU/ml for 4 days after drug treatment) on NK activity of normal MNC (see "Materials and methods" for details). Cytotoxicity assays were performed on day 4 after drug treatment. The results, expressed in terms of KC(10^6), are means of three independent experiments (*A* MNC not treated with cytokines, *B*, MNC treated with IFN, *C* MNC treated with IL-2). $^{**}P < 0.01$ according to Student's paired *t*-test analysis (see "Materials and Methods") for difference from corresponding controls

Results

In vitro studies

Preliminary in vitro studies have been performed to test the effect of the drugs used in the present clinical study on the NK function, which represents largely natural cell-mediated immunity in vitro [26]. MNC were incubated with HPC, 5-FU, MTX or HMF, alone or in combination with IFN or IL-2, as described in "Materials and methods". The results of the mean of three individual experiments, illustrated in Fig. 2, show that:

(1) treatment with HPC, 5-FU or HMF was moderately suppressive for the NK function of nonstimulated MNC, without, however, reaching statistically significant levels by either nonpaired or paired *t*-test analysis; (2) slight, but statistically significant inhibition of NKA was found in IFN-pretreated MNC exposed to MTX; (3) treatment with HPC or with HMF resulted in a significant reduction of NKA in IFN-pretreated MNC. Even stronger inhibitory effects provoked by HPC or HMF were detected in MNC exposed to IL-2 after drug treatment. However, it must be pointed out that treatment with IFN, and more evidently with IL-2, resulted in higher NKA than was observed in untreated MNC. This was observed not only in control lymphoid cell preparations, but also in MNC exposed to any of the three drugs or to the HMF combination.

Clinical studies

General toxicity

The results concerning general toxicity show that: (1) no treatment-related deaths or life-threatening toxicity were detected; (2) cytokine administration was followed by fever, chills, and moderate or minimal flu-like symptoms, muscle pain and weakness Antipyretic therapy with paracetamol was given for pyrexia higher than 38.5 °C, which occurred in all patients after cytokine administration during the first cycle of chemo-immunotherapy (3) gastrointestinal toxicity and mucositis were moderate in all patients; (4) mild erythema occurred at the injection site of IL-2 in all patients. The reaction usually developed after approximately 12 h and lasted for up to 48 h, mainly in the first and second cycles in all patients. In no case were pulmonary edema, hypotension requiring pressors or other symptoms attributable to the capillary leak syndrome [47] observed. No patient required hospitalization for toxicity directly related to IL-2 administration.

Blood chemistry studies indicate that kidney function was not significantly affected by the two therapeutic regimens, since no creatinine or BUN changes occurred in the patients during all treatment cycles (data not shown) and no liver toxicity was detected following drug treatment as evidenced by LDH, SGOT, SGPT, γ-GT, bilirubin and alkaline phosphatase values, which did not reach pathological levels in any case (data not shown). Urinalysis did not show any pathologic findings during the entire treatment period.

Hematological toxicity

No major changes in erythrocyte counts or hemoglobin titers occurred in all patients (data not shown).

The nadir WBC count was observed on day 15 of treatment for all patients in all cycles. Mean WBC counts declined from the initial value of 7000 cells/μl to approximately 4000 cells/μl. Significant recovery (approximately 6000 cells/μl) occurred on day 22, but only in chemo-immunotherapy cycles (i.e., after IL-2 administration, data not shown). Platelet counts were significantly reduced on day 15 in all cycles (from the initial mean value of 280 000 cells/μl to 240, 000 cells/μl for chemo-immunotherapy cycles, and 180 000 cell/μl for chemotherapy cycles). In any case, complete recovery was found on day 22 in all cycles (data not shown).

The kinetics of cell counts for lymphocytes (LYMPHO) polymorphonucleates (PMN), monocytes (MONO) and eosinophils (EOS) were analyzed separately, as illustrated in Fig. 3. (1) Significant reduction of LYMPHO was seen after both regimens, with the nadir on day 15. After IL-2 treatment (i.e., during chemo-immunotherapy cycles), the mean cell count not only showed a complete recovery, but reached a level significantly higher than that observed on day 1. On the other hand, cell count recovery was not detectable on day 22 in CMF cycles. (2) Significant declines of PMN counts relative to those of day 1 occurrred on day 8, 15 and 22 during both cycles. Moreover, cell number reduction on day 15 was slightly, although significantly, more pronounced during CMF cycles than during chemo-immunotherapy. (3) Significant reduction of MONO counts compared with those of day 1 occurred on days 8 an 15. On day 15 the decrease was marginally, although significantly, more pronounced during chemo-immunotherapy cycles than during chemotherapy cycles. On day

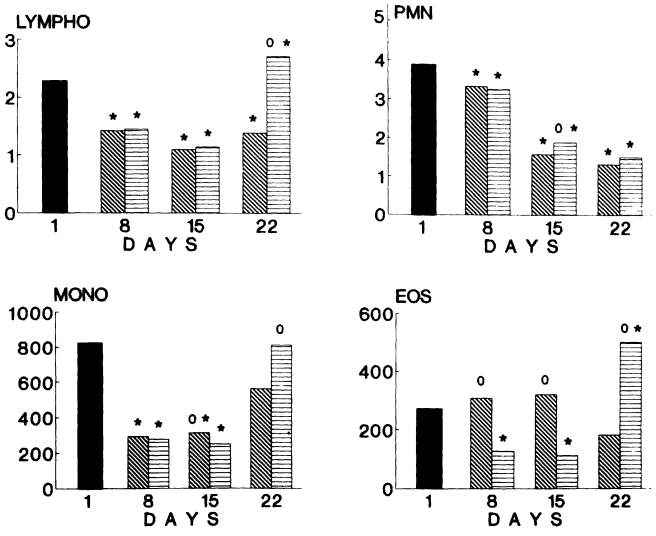

Fig. 3 Kinetics of cell counts specific to lymphoccytes (LYMPHO), polymorphonucleates (*PMN*), monocytes (*MONO*), and eosinophils (EOS) on days 1 (i.e. pooled data for cycles of chemo-immunotherapy and chemotherapy alone, *black columns*), and 8, 15, and 22 [i.e. during treatment cycles with CMF alone (*dashed columns*), or CMF + IL-2 + IFN (*hatched columns*)]. Blood cell counts are expressed as number of cells/μl (MONO, EOS), or as number of cells/μl $\times 10^3$ (LYMPHO, PMN). $*P < 0.05$ with respect to mean values on day 1 (see "Materials and methods"); o $P < 0.01$ (mean values for chemo-immunotherapy cycles vs those for chemotherapy cycles, on the same day)

22 cell count recovery was observed in the course of both cycles. In the chemo-immunotherapy cycles, when the patients had received IL-2 MONO counts on day 22 were significantly higher than those seen on day 22 during cycles of CMF alone. (4) All patients treated with chemo-immunotherapy showed a significant decrease in EOS counts on days 8 and 15. In contrast, during chemotherapy cycles, cell counts were significantly higher on days 8 and 15 than in chemo-immunotherapy cycles. On the other hand, the situation was reversed on day 22, when EOS counts were found to be significantly higher in patients pretreated with IL-2 during chemo-immunothrapy cycles than those detected in patients during cycles of chemotherapy alone.

A further analysis of hematological data was performed to evaluate cell counts for premenopausal and postmenopausal patients separately. Statistical analysis was carried out with Student's paired *t*-test. The results, not illustrated in detail in the present report, showed that the kinetics relative to LYMPHO or MONO counts were similar in pre and post menopausal patients and did not differ from those observed in all cases included in the "pooled" data of Fig. 3. PMN kinetics showed a trend to general decline during both chemo-immunotherapy and chemotherapy cycles, as shown for pooled data illustrated in Fig. 3. However, when PMN data for pre and postmenopausal patients were analyzed the decline was statistically significant on day 15 only for both types of treatment. EOS counts did not change significantly during chemotherapy cycles in pre or postmenopausal patients. When chemo-immunotherapy cycles were considered a significant increase in EOS count was detected in postmenopausal patients on day 22. A similar trend, not however reaching statistical significance was found in premenopausal patients. Therefore, the overall kinetics of EOS counts was essentially comparable to that observed for the pooled data illustrated in Fig. 3.

Statistical analysis relative to the hematological toxicity was also performed on all patients divided into two groups i.e. patients who started treatment with chemo-immunotherapy and patients who started treatment with chemotherapy alone. This was done to explore whether the "starting" cycle of treatment could have influenced the kinetics of the hematological parameters. This study did not reveal any major difference between the two groups (data not shown).

Immunotoxicology

Peripheral blood MNC were collected from all patients and tested for NKA. The results were calculated in terms of either KC(10^6), i.e., cytotoxic activity relative to fixed amount of effector cells, corresponding to 1 million cells), or KC(ml), i.e. number of target cells killed by effector cells presents in 1 ml of peripheral blood. Mean KC(10^6) values did not vary substantially between the two types of treatment during the entire period of adjuvant therapy (data not shown). When the natural cytolytic activity was analyzed in terms of KC(ml), the results relative to pooled data of all cycles, illustrated in Fig. 4A, show that the kinetics of this immune function was obviously also dependent on the number of LYMPHO/ml. A significant decrease in natural immune reactivity was detectable on day 15, when the nadir of the LYMPHO count occurred. Complete recovery of NKA was found after IL-2 (day 22) or IL-2 + IFN (day 23) administration in all chemo-immunotherapy cycles. Modest but significant increases of natural cytotoxicity were detected on days 22 and 23 during all cycles of chemo-immunotherapy (i.e. after IL-2 and IFN) when mean NKA was compared with "basal" NKA (i.e. cytolytic activity of MNC measured before entering into the study). Mean basal NKA of all patients was as follows: mean KC(10^6) = 1343, SEM = ±469; mean KC(ml) = 3055, SEM = ±411. These basal values were significantly lower ($P < 0.05$) than those found on day 22 [KC(ml) only; see Fig. 4A], or on day 23 [KC(10^6), mean = 2424, SEM = ±409, and KC(ml); see Fig. 4A].

Further analysis of KC(ml) values was made to explore possible "carryover" effects of IL-2 + IFN pretreatment (performed during chemo-immunotherapy cycles) on the natural immunity pattern in the course of chemotherapy cycles. In this case, the KC(ml) values considered were those pertaining to: (1) first cycle, day 15 [i.e. before treatment with IL-2 + IFN, for A (chemo-immunotherapy) and B (chemotherapy) groups, which were pooled]; (2) first cycle, day 23 (i.e. after immunotherapy for group A, or after no treatment for group B); (3) second cycle, day 1; (4) second cycle, day 15. The results, illustrated in Fig. 4B, show that natural cytotoxicity was significantly higher in the group subjected to chemo-immunotherapy than in the group treated with chemotherapy alone, on day 23 of the first cycle. On day 15 of the second cycle no significant difference was detectable between KC(ml) values of group B and those of group A. It should be stressed that group A had been treated with IL-2 + IFN during the first cycle. In contrast, group B was not exposed to cytokines, either during the first cycle (in which it was treated as group A), or during the first 15 days of the second cycle. This result suggests that the effect of treatment with IL-2 + IFN was short-lived and did not influence the natural immune function in the course of subsequent alternating cycles of chemo-immunotherapy and of chemotherapy alone. Further support for the validity of the experimental design adopted in the present and in the previous investigations [13, 14] stems from pharmacokinetic studies on IL-2, performed by Banks et al. [3] in patients with renal cell carcinoma treated with IL-2, IFN and 5-FU. This investigation showed that following subcutaneous injections of low doses of IL-2, plasma levels of the cytokine peaked at 12–18 h, tending to return to normal values by 48 h into the treatment, and approximately 60 h after the last injection of the agent.

Cytofluorimetric analysis was performed to evaluate the percentages of CD16+ and CD25+ cells among the

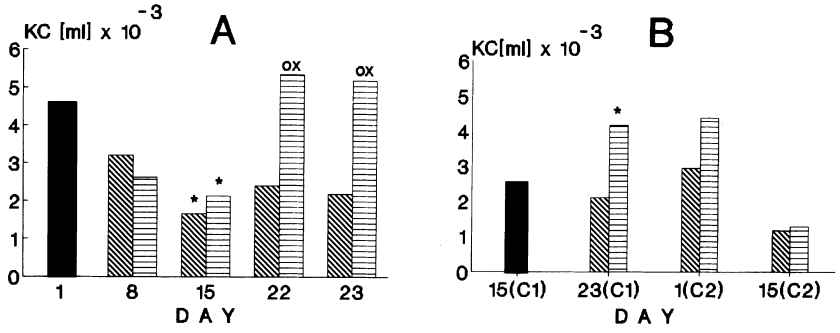

Fig. 4A,B NKA in the peripheral blood of patients. Data are expressed in terms of mean KC(ml), i.e. number of target K562 cells killed by effector cells present in 1 ml of peripheral blood. This is a measure of the absolute cytolytic activity detectable in a volume unit of peripheral blood. For the cytolytic assay see 'Materials and methods.'' **A** Kinetics of NKA on day 1 (i.e. pooled data for cycles of chemo-immunotherapy and chemotherapy alone, *black columns*) and days 8, 15, 22, and 23 [i.e. during treatment cycles with CMF alone (*dashed columns*), or CMF + IL-2 + IFN (*hatched columns)*]. $^{*}P < 0.05$ (mean values on day 1 vs those on other days of the cycle); $^{x}P < 0.01$ (mean values on day 15 vs those on day 22 or 23); o $P < 0.01$ (mean values for chemo-immunotherapy cycles vs those for chemotherapy cycles on the same day). **B** NK activity in the peripheral blood of patients subjected to chemotherapy alone (*dashed columns*), or to chemo-imunotherapy (*hatched columns*), on day 15 [15(C1), pooled for the two treatment groups, *black column*], on day 23 [*23(C1)*] of the first cycle, or day 1 [*1(C2)*], or day 15 [*15(C2)*] of the second cycle $^{*}P < 0.05$ for mean values for chemo-immunotherapy on day 23(C1) vs that for chemotherapy alone on day 23(Cl), or vs that of pooled chemo-immunotherapy + chemotherapy on day 15C1, or vs both mean values on day 15C2

peripheral MNC (Fig. 5). This was done to detect the possible influence afforded by the two regimens on the number of activated NK cells (i. e. CD16+ cells) [32] and on the number of MNC expressing the alpha chain of IL-2 receptor (IL-2R) that is induced on activated T-lymphocytes [58]. the results, illustrated in Fig. 5A, B, show that (1) chemotherapy alone produced a progressive and longlasting decrease in the number of CD25+ MNC, whilst significant recovery of CD25+ cells occurred after IL-2 or IL-2+IFN treatment (days 22 and 23, respectively, of chemo-immunotherapy cycles); (2) similar cell kinetics was found for CD16+ lymphocytes; (3) no change in the number of CD4+ and CD8+ T cells was detected during either treatment cycles (data not shown).

A further analysis of immunotoxicological data was also performed on premenopausal and postmenopausal patients separately. The results did not substantially differ from those observed with pooled data (data not shown).

Discussion

The aim of the present pilot study was to explore the feasibility of a new approach to the adjuvant therapy of breast cancer, based on a chemo-immunotherapy regimen. This consisted of classic chemotherapy with CY + MTX + 5-FU, followed by low-dose IL-2 and IFN. Treatment with low-dose IL-2 was previously found to be clinically effective with modest systemic toxicity [23, 32, 47, 58]. Moreover the cytokine regimen has been designed to utilize the immunomodulating properties of IL-2 and IFN, and could be also useful for possible direct detrimental effects of IFN on cancer cells.

The rational bases for adopting sequential treatment with IL-2 and IFN derive from previous investigations, carried out in our laboratory, concerning the effects of IFN and IL-2 on NKA of human MNC of different donors [18]. It was found that treatment with IFN must be performed after rather than before exposure of MNC to IL-2. This schedule provides the best chances of inducing a higher lytic activity than can be obtained after treatment with IL-2 alone [18]. Moreover, it was found that CMF chemotherapy is able to depress the NKA of peripheral blood MNC in breast cancer patients [52]. In the same study, beta-IFN antagonized the depressive effects of chemotherapy on the NK function, either in vivo or in vitro. These findings, along with previous observations showing a good relationship between in vitro and in vivo effects of beta-IFN on NKA [12], encouraged us to design a chemo-immunotherapy protocol, based on an IL-2 + IFN combination added to antineoplastic agents routinely used in adjuvant chemotherapy.

Preliminary in vitro experiments have been conducted to obtain immunopharmacological data, which are not available from the literature, on the possible interaction between cytokines, such as IFN or IL-2, and the drugs included in the classic adjuvant chemotherapy for breast cancer (i.e. CMF). The results of this investigation indicate that the in vitro-active CY derivative HPC, and HMF noticeably antagonize the effects of either IFN and IL-2 on NI (Fig. 2).

It is known that 5-FU and MTX are phase-specific agents [9]. Therefore, since NI effector cells are mainly in G0 [11], these drugs are not expected to affect the NI function, substantially, as confirmed by the results illustrated in Fig. 2. On the contrary, in the case of MNC stimulated by IL-2, IL-2R+ cells are driven into cell cycle by the lymphokine, and should be susceptible to the suppressive effects mediated by phase-specific agents. However, in this case, similarly to the clinical situation (see below), MNC were exposed to the drugs for a limited period of time (i.e. 1 h). It follows that, when IL-2R+ MNC involved in NI were stimulated by IL-2, no

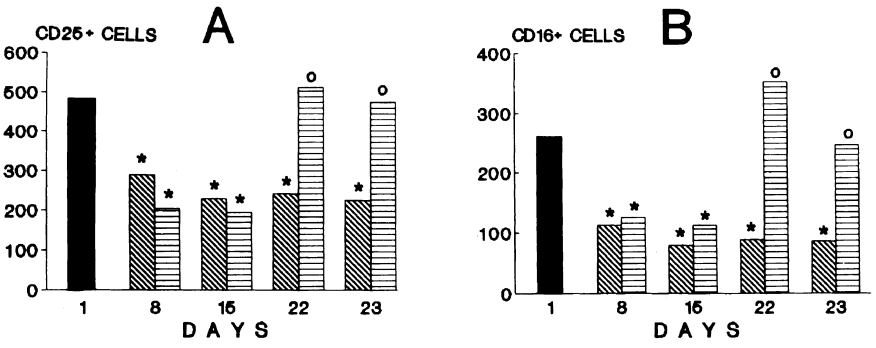

Fig. 5 Lymphocyte subpopulations (**A** CD25 + cells, **B** CD16+ cells) detected in patients during chemo-immunotherapy or chemotherapy cycles. Phenotypic analysis of MNC was performed on day 1 (i.e. pooled data for both chemo-immunotherapy or chemotherapy cycles, *black columns*), and on days 8, 15, 22, and 23 during chemotherapy cycles (*dashed columns*) and chemoimmunotherapy cycles (*hatched columns*). Data are expressed in terms of number of positive cells per microliter of peripheral blood. $^{*}P < 0.05$ (mean for day 1 vs means for other days of the cycle), $^{o}P < 0.01$ (mean for chemo-immunotherapy cycles vs means for to chemotherapy cycles on the same day)

sufficient drug concentration was presumably available to influence cell growth and effector function. Moreover, a number of antitumor agents, including 5-FU and MTX, are cytotoxic because they induce apoptotic death [29]. Since IL-2 was found to upregulate bcl-2 in NK cells and protect them from apoptosis [2], it is reasonable to hypothesize that a similar mechanism could be involved in the interaction between IL-2 and the two antimetabolites under study.

Actually the results of Fig. 2 stress that treatment with HPC or HMF is followed by a significant decline in NKA, and 5-FU and MTX do not make a significant contribution to the inhibitory effects of HPC. In any case, these results indicate that the combined treatment with cytokines and chemotherapy, including HMF association, resulted in much higher NKA than that detectable in untreated control MNC. Therefore, the preliminary in vitro investigations described here provide further support for the rationale according to which the present clinical study was designed.

Adjuvant treatment of breast cancer patients with alternating cycles of chemo-immunotherapy and chemotherapy did not provoke any major toxic effects on different organs. The absence of cardiovascular and pulmonary disorders, which have been described during high-dose IL-2 administration [45], is in line with the results of previous studies showing that the lymphokine is essentially nontoxic at the dose levels utilized in the present study [23, 32, 47, 58].

The kinetics of blood cell counts referred to drug and cytokine treatment shows a common pattern of IL-2-dependent recovery for whole WBC (data not shown), LYMPHO, MONO and EOS (Fig. 3). These results, similar to those reported from previous clinical trials using IL-2 [10, 47], are consistent with the presence of functionally active IL-2 receptors on LYMPHO [49], MONO [25, 36] and EOS [46], responding to in vivo administration of low doses of the lymphokine. In contrast, IL-2 administration did not influence platelet recovery, as shown by platelet counts in the course of both types of treatment on day 22 (data not shown).

In spite of the recently demonstrated presence of functionally active IL-2 receptors on polymorphonucleates (PMN) [19], treatment of patients with the lymphokine did not result in early recovery of PMN counts, as illustrated in Fig. 3. No data presently available make it possible to understand the mechanism underlying the lack of IL-2 activity on this parameter.

The immunological effects of low-dose IL-2 + IFN on NI have been demonstrated by the kinetics of NKA analyzed in terms of KC(ml) values, as illustrated in Fig. 4A. The results confirm previous clinical observations, indicating that even low doses of the lymphokine are capable of producing significant immunological effects [23, 32, 47, 58]. As expected on the basis of cell count analysis, the influence of IL-2 on NKA is more evident when the blood concentration of LYMPHO is taken into account [i.e. when KC(ml) values are considered]. Actually, KC(ml) is a measure of the cytolytic properties of 1 ml of peripheral blood, thus providing an estimate of the cytotoxic activity of circulating MNC. These results are supported also by the analysis of the total number of CD25+ and CD16+ cells. In fact IL-2 treatment was followed by significant increase of the number of cells expressing the IL-2R alpha chain, actively responding to IL-2 itself [55]. If NI is specifically considered, the significant increase of CD16+ cells is in line with the increment in NKA expressed in terms of KC(ml).

In conclusion, the present study demonstrates that low doses of IL-2 along with low doses of IFN promote recovery of the normal population of lymphoid cells associated with only minor toxicity in patients operated on for early breast cancer. However, further randomized studies on an adequate number of patients are required for any comparative analysis on the protective effects of CMF alone compared with those of the same drugs associated with the two cytokines in adjuvant therapy.

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