Introduction

Breast cancer results in ~40,000 deaths yearly, making it the second leading cause of death from cancer in women (1). The rate of death from breast cancer has remained relatively unchanged from before 1940 until the mid-1990s, since that time it has decreased by 2.3% yearly (1, 2). Metastatic breast disease, at time of diagnosis, is a negative prognosis indicator (e.g., the risk of death for women with three or more positive lymph nodes is ~25% greater than that for patients with similar-sized tumors but negative lymph nodes; ref. 3). Currently, metastatic breast cancer is treated with either hormones or cytotoxic drugs. More aggressive, highly invasive tumors are generally less responsive to either therapeutic approach. The main cytotoxic chemotherapy regimens include the use of doxorubicin, fluorouracil, cyclophosphamide, and taxanes (4–6), but their usage is limited by toxicity. New treatments, such as immunotherapies, capable of combining high efficacy against metastatic breast tumors with low toxicity are needed to significantly affect the prognosis of patients with this disease.

Although cytotoxic chemotherapeutic agents have traditionally been thought of as immunosuppressive, there is considerable literature that shows that many chemotherapeutic agents, including doxorubicin, can stimulate antitumor immune responses under certain conditions (7, 8). One or two injections of doxorubicin at moderate doses increase the activity of macrophages, natural killer (NK) cells, lymphokine-activated killer (LAK) cells, and CTLs and increase the production of interleukin-2 (IL-2) both in mice and in humans (7–10).

IL-2 at very high doses has been shown to have limited clinical efficacy against certain tumor types (9, 10), but it is not clear that immune modulation is involved. At lower, more physiologic doses, IL-2 has been shown to stimulate potential antitumor immune responses, such as proliferation and differentiation of activated T and B cells, increased IFN-γ production, activation of NK cells, and induction of LAK cells (11). The effects of IL-2 on T cells promote a Th1 response, and LAK cells, NK cells, and/or CTLs have been implicated in IL-2-induced antitumor responses (12).

Generation of therapeutically effective antitumor immune responses with induction of antitumor immune memory was found to be dependent on the dose and schedule of both doxorubicin and IL-2 and required prolonged IL-2 administration (13–15). Treatment of C57BL/6 mice bearing the EL4 syngeneic T-cell lymphoma with a single moderate dose of doxorubicin followed by twice daily injections of a low-dose IL-2 for 30 days resulted in up to 80% tumor-free survival; progressive disease was seen when treatment was either IL-2 or doxorubicin alone (13). The curative treatment was ineffective in mice depleted of CD8 T cells (13, 14). The combination treatment was equally effective in mice bearing either doxorubicin-sensitive or 10-fold doxorubicin-resistant EL4 tumors, this establishing that major direct tumor debulking by doxorubicin was not required. Surviving mice were shown to have anti-EL4 immune memory for as long as 2 years after treatment (13, 15).

The characteristics of the effects of the combination of doxorubicin plus IL-2 against established E0771 medullary breast adenocarcinoma are reported herein. E0771 tumors, implanted s.c. in syngeneic C57BL/6 mice, are immunosuppressive and highly aggressive, invading locally into dermal layers and the peritoneum as well as distantly to the lung and have characteristics that closely mirror those of the human disease (16). The data presented herein show that doxorubicin plus IL-2 immunomodulation-based antitumor therapy is curative in this mouse model of breast cancer.

Materials and Methods

Cell Lines

E0771 mouse breast cancer cells were obtained from Dr. F.M. Sirotnak (Memorial Sloan-Kettering, New York, NY) and maintained in RPMI 1640
supplemented with 10% calf serum with Life Technologies, Grand Island, NY) and 10 mmol/L HEPEs. YAC-1 mouse lymphoma cells and EL4 cells [available from American Type Culture Collection (ATCC), Rockville, MD] were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS and 10 mmol/L HEPEs. Lewis lung carcinoma cells (ATCC) were maintained in DMEM supplemented with 10% heat-inactivated FCS. Culture incubation conditions were 37°C, 100% humidity, and 5% CO₂.

Animals
Female C57BL/6 mice were obtained through the National Cancer Institute (NCI) grantees program (Frederick, MD). Mice were kept in sterilized filter top cages with controlled humidity, 12-hour day/night cycles at 22°C. LM-485 rodent chow (Harlan Teklad, Madison, WI) and water were provided ad libitum. Experiments were started when mice were 8 to 12 weeks old.

Agents
Recombinant human IL-2 was a gift from Chiron (Emeryville, CA) and diluted in sterile 0.3 mol/L glucose. Doxorubicin (a gift from Adria Laboratories, Columbus, OH) was diluted in sterile water and protected from light exposure.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay
The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) 96-well plate assay was used to determine the relative amounts of cells in wells after exposure to various agents. The procedure was as follows: day 1, plated 2 × 10⁵ cells in 200 µL/well; day 2, centrifuged plates (350 × g, 5 minutes), removed 100 µL medium, and added II-2 or medium (50 µL/well) together with a concentration gradient (seven half-log steps, starting at 1.7 mmol/L) of doxorubicin (50 µL/well). All assays included appropriate controls. On day 5, a standard MTT dye (Sigma-Aldrich, St. Louis, MO) enzymatic reduction assay was done (17).

Cancer Cell Injections
Cultured E0771 and Lewis lung carcinoma cells were suspended by trypsin digestion (0.05% trypsin-EDTA, 5 minutes). Cells were washed thrice with HBSS, counted, and diluted in this solution and 2.5 × 10⁴ cells in 200 µL were injected s.c. in the lower abdomen of each mouse in or near the no. 4 mammary fat pad. Day of the implantation of the tumor cells was designated day 0.

Tumor Measurement
The tumor's greatest dimension and the one perpendicular to it were measured every 2 to 3 days using dial calipers and expressed as length × width = tumor size.

Drug and Antibody Injections
In 1v. procedures were done while mice were restrained in a plastic mouse holder. Mice received a single i.v. injection of doxorubicin (5 mg/kg) usually on day 8. IL-2 (100,000 IU/injection) was given i.p. twice daily starting the day after doxorubicin administration and continued for 31 days.

The following antibodies were given to deplete specific subsets of immune cells: anti-mouse CD8α, anti-mouse CD4 (BD PharMingen, San Diego, CA), or ascites fluid generated using the hybridoma cell lines (ATCC) in nude mice [TIB210, clone 2.43 (anti-mouse CD8α) and HB191, clone PK136 (anti-mouse NK1.1)]. Antibodies (ascites 5 µL/injection or commercial antibodies 16 µL/injection) were injected i.p. on days –2, –1, 10, 11, 20, 21, 30, and 31. In those experiments involving antibody switching (e.g., anti-CD8α then anti-NK1.1), the first of the pair of antibodies was given on days –2, –1, and 10 and the second was given on days 20, 21, 30, and 31.

Immune Cell Cytotoxic Activity Assay
The effects, relative to control groups, of different treatments on immune effector cell cytolytic activities of splenocytes from mice bearing E0771 tumors were measured ex vivo using reported procedures (14, 15, 18). Briefly, splenocytes (5 × 10⁶/100 µL/well) were plated in 96-well plates, six replicate wells for each experimental variable. For LAK cytolytic activity, splenocytes were incubated for 5 days in effector stimulation cultures with IL-2 (5.2 × 10⁸ IU/100 µL/well). For CTL activity, splenic effector cells were stimulated in 5-day cultures with lethally X-irradiated E0771 stimulator cells added to give splenocyte to stimulator cell ratios of 1:0 (i.e., minus stimulator control), 50:1, and 100:1. For NK cytolytic activity, plated splenocytes (effectors) were assayed without stimulation.

To assess the level of cytolytic activity, the plated effector cells (either immediately or after 5-day cultures) were serially diluted (2-fold) four times. Chromium 51 (Perkin-Elmer, Boston, MA)–labeled target cells (E0771 for LAK and CTL activities and YAC-1 for NK activity) were added at 5 × 10³ per 100 µL to the diluted effector cells to generate ET ratios of 100:1, 50:1, 25:1, and 12.5:1. Maximum (Triton X-100 detergent) and spontaneous (growth medium) ⁵¹Cr release control groups were always included. Following a 4-hour incubation, the radioactivity in 100 µL cell-free aliquots was measured using a Packard Instrument (Meriden, CT) auto-gamma counter. The percent specific release of ⁵¹Cr from the target cells, indicating the amount of cytolytic activity of the effector cells, was calculated with the following equation:

\[
\text{Percent specific release = } \frac{( \text{average } \text{ER} - \text{average SR})}{\text{average } \text{MR} - \text{average SR}} \times 100\%
\]

where ER is experimental release, SR is spontaneous release, and MR is maximum release.

Flow Cytometric Analysis
Reported procedures were used to stain cells with fluorescent-labeled antibodies to assess the expression of specific surface proteins (17). Reagents included the following: blocking reagent: mouse γ-globulin (25 mg/mL, Cappel, Cochranville, PA) plus bovine serum albumin fraction V (16 mg/mL, Roche, Indianapolis, IN) in PBS; cell-surface detecting antibodies: phycoerythrin (PE)-anti-mouse NK1.1, FITC-anti-mouse CD8α,2 (BD PharMingen), PE-anti-mouse CD8α, PE anti-mouse CD4 (Caltag, San Francisco, CA); isotype control antibodies: hamster FITC-IgG2a, rat FITC-IgG2a, mouse PE-IgG2a, mouse FITC-IgG1α, (BD PharMingen), and mouse FITC-IgG2α (Caltag).

Metabolic Analysis
¹H nuclear magnetic resonance spectroscopic analysis of the serum samples. Blood samples were obtained by cardiac puncture of deeply (stage IV) anesthetized mice. Serum was collected and stored at –80°C until analyzed. The serum samples were thawed immediately before use, and 100 to 200 µL of each (depending on availability) were diluted with 99.9% D₂O to a total volume of 600 µL in 5-mm precision nuclear magnetic resonance (NMR) tubes (Norell, Landisville, NJ) to provide field frequency lock. ¹H chemical shifts were referenced internally to the residual water (HOD) resonance at 4.9802 measured relative to the primary internal chemical shift reference trimethylsilyl-2,2,3,3-tetradeuteropropionic acid at 0.00. Conventional ¹H NMR spectra of the serum samples were measured on a Bruker AMX-600 spectrometer (Billerca, MA) operating at a frequency of 600.13 MHz ¹H at 278K. To suppress the large water signal, the ¹H NMR spectra were acquired using a pulse sequence called NOESYPRID, comprising the following pulse sequence: RD – 90° – t₁ – 90° – tₐm – 90° – acquire free induction decay (FID), where RD represents a relaxation delay of 1.5 seconds during which the water resonance is selectively irradiated; t₁ represents the first increment in a NOESY experiment and corresponds to a fixed interval of 4 µs; and tₐm is the mixing time in the NOESY sequence and has a value of 100 ms, during which the water resonance is again selectively irradiated. For each sample, 128 FIDs were collected into 64K data points using a spectral width of 12.2 kHz and an acquisition time of 2.69 seconds. The FIDs were multiplied by an exponential weighting function corresponding to a line broadening of 0.25 Hz before Fourier transformation.

Data reduction of NMR data. Each ¹H NMR spectrum was phased and baseline corrected using NutsPro version 20021122 (Acorn NMR, Inc., Livermore, CA) and the 9.5-0.0 ppm spectral region was reduced (bucketed) to 219 integral segments of equal width of 0.04 Hz. This optimal width of segmented regions is based on previous studies (19, 20), which found that regions of 0.04 ppm accommodated any small pH-related shifts in signals
and variation in shimming quality. To eliminate any variability in the suppression of water resonance, that region (65.5-4.75) was eliminated. Subsequently, all remaining buckets of the spectra were normalized to the total integrated area of the individual spectrum.

**Principal component analysis of the 1H NMR spectra.** Principal component analysis (PCA) is an unsupervised multivariate statistical method (i.e., analysis done without use of knowledge of the sample class) that reduces the dimensionality of the data by a “grouping” of variables (metabolic signals) that have strong correlations with one another into a smaller set of variables known as principal components, which in turn represent a percentage of the variation in the data. The components themselves are not intercorrelated and thus represent distinct patterns of metabolic signals. The variables associated with a specific component produce loadings that are quantified as the correlation of that variable with the variance represented by that component. Individual buckets are then assigned a score for each component calculated as the sum of the weighted factor loadings for each factor. PCA was carried out using Pirouette version 3.10 (Infometrix, Inc., Bothell, WA) on the 1H NMR data from the sera that were first mean-centered and auto-scaled. The results were plotted three-dimensionally to indicate relationships between samples in the multidimensional space.

**Statistical Analysis**

The drug efficacy and immunologic activity data were analyzed initially by simple descriptive statistics of mean and SD. Minitab version 13.2 statistical software (Minitab, Inc., State College, PA) was used for the statistical analyses. The significance of the difference between the means of two variables was determined by a two-tailed t test at a 95% confidence level.

**Results**

**Effect of doxorubicin plus IL-2 on anti-E0771 tumor response and survival.** A complete response was defined as no detectable tumor 60 days after tumor cell injection (i.e., ~20 days after treatment ended), and a partial response was defined as delayed tumor growth relative to that of the control group. Treatment of mice bearing E0771 tumors with doxorubicin plus IL-2 induced both complete and partial responses (Fig. 1A). A complete response occurred in 40% and a partial response in 30% of the mice (pooled data from four experiments). There were no complete antitumor responses in untreated mice (Fig. 1A and B) or mice treated with only doxorubicin or IL-2 (Fig. 1A). All animals initially had detectable tumor (10-20 mm² by days 11-15). However, in mice showing a complete response, the tumors never grew to a large size and were undetectable before the tumors in control animals had reached the predetermined size (2 cm in the greatest dimension, Institutional Animal Care and Use Committee regulation) requiring host sacrifice. Mice whose tumors had become undetectable did not relapse (250 days maximum follow-up).

**Specific resistance of mice “cured” with doxorubicin plus IL-2 to reimplantation of E0771.** Mice that had a complete response were reinoculated with E0771 (5 × 10⁶ cells s.c.) on day 74 and again on day 121 after the initial E0771 cell injections, and tumor growth was compared with that of naive mice inoculated in parallel (Fig. 2). Mice that had a complete response remained tumor free following E0771 cell reimplantation, whereas tumors in each of the naive control mice grew to a size that required euthanasia. When the data from three independent experiments were pooled, 83% of the complete responder mice tested were found to be resistant to E0771 on tumor reimplantation. To test the specificity of this resistance, 13 mice with shown ability to rejet reimplanted E0771 were inoculated with cells of a non-E0771 cross-reactive C57BL/6 syngeneic tumor (either EL4 lymphoma or Lewis lung carcinoma cells) and tumor size was measured every 2 to 3 days. In 12 of the 13 mice, tumors grew progressively (data not shown).

**Doxorubicin plus IL-2-induced toxicities.** Body weight of the mice was monitored during the period of treatment and immediately afterward as an indication of toxicity. In three experiments, a total of 192 mice received doxorubicin plus IL-2 treatment with or without monoclonal antibody treatment to deplete specific lymphoid cell subsets. In ~95% of the mice, a 1% to 2% weight gain with respect to pretreatment weight was observed; <5% of the mice lost >5% of their pretreatment weight and only 1.5% of them (i.e., 3 of 192) lost ≥10% (data not shown).

**In vitro effect of doxorubicin plus IL-2 on the growth of E0771 cells.** To determine if IL-2 is either directly cytotoxic to E0771 cells or enhances their sensitivity to doxorubicin, the effect of IL-2 alone and of IL-2 plus doxorubicin on E0771 cells was determined in culture. E0771 cells were cultured with IL-2 (0.05, 125, or 20,000 IU/mL) alone or in combination with doxorubicin at seven concentrations from 0.001 to 1 μg/mL and the relative numbers of viable cells were assessed 3 days later. The highest concentration of IL-2 used was greater than the predicted systemic level achieved in mice receiving the treatment protocol.

![Figure 1: Therapeutic effectiveness of the treatments studied.](image-url)
Doxorubicin induced the expected concentration-dependent cell growth inhibition [i.e., in the MTT assay, there was a linear decrease in the absorbance (0.700-0.05 absorbance) at 570 nm over the concentration range of 0.01-1 µg/mL doxorubicin]. The addition of IL-2 did not significantly alter E0771 cell growth or their sensitivity to doxorubicin.

Effect of treatment on leukocyte cytolytic activity. The cytolytic activities of LAK, CTL, and NK cells in splenocyte populations of mice from the different treatment and control groups were measured ex vivo. Although in the prior EL4 study LAK cell involvement had been ruled out, it was considered prudent to examine them in a study involving long-term twice daily IL-2 treatment. Before and weekly during treatment, leukocyte cytolytic activities were assessed. Splenocytes from each animal (three to five mice per treatment group) were evaluated separately and data from replicate animals within a single group were pooled (Fig. 3). No significant differences were seen between the LAK cytolytic activities from tumor-bearing treated mice and naive mice at any time (Fig. 3A). Six weeks after the injection of E0771 cells, however, when tumor burden was high in the untreated tumor-bearing mice, their LAK cytolytic activity was very low compared with that of the cells from naive non-tumor-bearing mice or those receiving IL-2 (0% and 60%, respectively). CTL activity increased significantly in the treated mice (3-29% between weeks 3 and 6), whereas in untreated mice it decreased, although not significantly (Fig. 3B). NK cytolytic activity (Fig. 3C) was significantly higher in treated mice than in untreated or naive mice on weeks 2 to 4 (maximum difference was seen on week 3), but later (weeks 5 and 6) it returned to background (naive) levels. During weeks 4 to 6, the activity in the cells from the untreated tumor-bearing mice was significantly lower than that of naive mice.

E0771 tumor-induced immune suppression. Because immune cell cytolytic activity decreased in untreated E0771 tumor-bearing mice (Fig. 3), the potential immunosuppressive effect of cells from mice with progressive disease was investigated by examining how the inclusion of splenocytes from those mice affected generation of anti-E0771 CTL activity in culture (Fig. 4). The effector cells were from mice exhibiting an antitumor response following treatment with doxorubicin plus IL-2. The methodology was the same as that of the CTL generation cultures, except that, in addition to effector splenocytes, 1% or 10% additional splenocytes to be tested for putative suppressor activity were added (20). In addition to these mixtures, each splenocyte population was assessed alone for generation of anti-E0771 activity. The effector cells alone displayed a strong anti-E0771 response, but none of the suppressor cell populations alone developed any cytolytic activity. When combined, splenocytes from untreated mice bearing E0771 tumor significantly suppressed the stimulation of the anti-E0771 CTL activity of the effector splenocytes. The addition of 10% suppressor cells completely inhibited the generation of CTL activity and the addition of 1% had a lower but significant \((P < 0.001)\) effect. Splenocytes from treated nonresponding mice also significantly decreased generation of CTL activity but to a much lesser extent than did those from untreated mice. Splenocytes from naive mice did not significantly decrease CTL activity, which indicates that the decreased activity seen with the addition of the other two splenocyte populations was not due to either the increased cell density or the slight dilution of cells.

Immune cell requirements for the effects of doxorubicin plus IL-2 on E0771 to occur. To examine the contribution of specific lymphocyte subsets to the curative doxorubicin plus IL-2 treatment, mice were injected, both before E0771 tumor inoculation and throughout therapy, with antibodies against cell-surface markers (CD4, NK1.1, and CD8) to deplete specific cells (Fig. 5). Among groups that did not receive antibodies and were treated with doxorubicin plus IL-2, ~45% of the mice achieved a complete response and 10% had an increased life span. Animals treated with anti-CD4 antibodies were shown, by flow cytometric analysis 5 days after the second of the two injections, to have ~66% less CD4 T cells than control mice. Nevertheless, the percentage of complete responses was not significantly changed in that group (data not shown). Treatment of the mice with anti-NK1.1 antibody completely eliminated NK cell cytolytic activity as assessed ex vivo (data not shown). Depletion of NK1.1 cells reduced the percentage of complete responses (i.e., from 45% to 30%); this effect, however, was not statistically significant \((P > 0.5)\). When NK1.1 cells were depleted early followed by depletion of CD8 T cells late during therapy (data not shown), the percentage of mice with a complete response was decreased compared with that seen in the group depleted of NK1.1 cells throughout treatment (10% versus 30%, respectively). Either anti-CD8a or anti-CD8b,2 antibodies completely eliminated the respective specific cell subsets as determined by flow cytometry and cytolytic activity assays (data not shown). Antibody depletion of CD8a cells either early (i.e., only days −2, −1, and 10, and 11; data not shown) or throughout therapy (Fig. 5) completely eliminated any response to treatment. Similar results were obtained when CD8b,2 cells were depleted (data not shown).

Relationship of serum biomarker to treatment-induced responses. Initial observations were made toward determining NMR detectable patterns of blood-borne organic molecules (small-molecule metabolites) that might serve as surrogate end points of response to therapy. Sera were collected from mice in four experimental groups (naive mice, mice with shown resistance to E0771 inoculation, and either mice 16 to 32 days after treatment started that were responding to treatment or that had
progressive disease). NMR spectra were obtained from each serum sample and processed as described in Materials and Methods. PCA analysis of the NMR data was used to detect any possible patterns in metabolite profile variation across the experimental groups. Each sample was assessed for correlations with response to treatment/condition. Subsequently, the three factors (principal components) that account for the highest variation among samples were chosen, and each individual serum sample was plotted against a three-dimensional coordinate system based on these factors (Fig. 6A). This figure shows that factor 2 can be used to separate the mice into three groups. Those high in serum components that compose the second principal component (furthest from the axes origin) consist of mice that responded to treatment. A second group with low abundance of components that comprise factor 2 consists of mice that did not respond to treatment. Naive mice showed intermediate level of components in factor 2. This suggests that the components of factor 2 might predict response to treatment.

To determine if factor 2 could predict response to treatment before response could be determined by decreasing tumor size, two sets of mice were used. In one set (donor groups), mice were treated and used to collect blood for metabonomics analysis, and in another set (monitored groups), mice were treated in parallel but were observed for anti-E0771 tumor response. In a single experiment, blood was collected from the donor mice just 10 days after initiation of treatment. The sera were analyzed metabonomically as done previously. The response of the mice, at the time the blood was collected by terminal cardiac puncture, was not yet clinically determinable. Therefore, the results with the individual blood samples were categorized by the response seen in the similarly treated monitor mice (10 mice per group). Approximately 60% of the serum samples from donor mice within groups for which mice in the parallel monitor groups went on to achieve complete responses had elevated levels of metabolites that compose factor 2 as shown by their position on the corresponding axis (Fig. 6B). Sera from donor mice from groups in which none of the monitor mice achieved a complete response generally had lower concentrations of factor 2 components. Most nonresponders that showed an increase in factor 2 components could be separated from responders based on contributions from other principal components. Naive mice again showed intermediate levels of factor 2 components in their sera. These results are consistent with the possibility of using components from factor 2 as markers for predicting response.

Preliminary analyses of some of the components that make up factor 2 have been completed. In both experiments, major contributor metabolites with resonances having peaks from 0.83 to 0.89, 1.01 to 0.97, and 3.21 to 3.17 were observed. Identification of the metabolites, based on literature values, indicate that very low density lipoprotein, valine CH₃, and β-glucose are the most likely candidates for each peak, respectively. Indeed, there are other lesser components of factor 2 at 3.62 and 2.29, which correspond to valine resonances, as well as components at 4.62, 3.74, 3.90, and 3.41 that contain β-glucose resonances. Final assignment must await experimental confirmation.

**Discussion**

The efficacy of the doxorubicin plus IL-2 treatment against the highly invasive metastatic (16) mouse E0771 breast medullar adenocarcinoma was evaluated based on the rate of response, increased life span, and ability of cured mice to survive cancer rechallenge. The doxorubicin plus IL-2 combination treatment eliminated tumor in 40% and delayed tumor growth in 20% of the mice, all of which had palpable tumor at initiation of treatment without significant toxicity. The mice that achieved a complete response remained tumor free (250 days maximum follow-up) and therefore were effectively cured. As in the EL4 lymphoma model (13), only treatment with the doxorubicin plus IL-2 combination therapy was curative and neither doxorubicin alone nor IL-2 alone had any detectable effect. Those animals that had a response to treatment generally exhibited a decline in tumor growth ~11 to 13 days after the beginning of treatment and tumor regression, during
The data indicate that the curative effect of doxorubicin plus IL-2 treatment is mediated by an anti-E0771 CTL response. When splenocyte cytolytic activities were measured ex vivo, only CTL and NK cell, but not LAK cell, activities increased during doxorubicin plus IL-2 treatment, although treatment did sustain LAK activity at control level in the late stages of the disease. The CTL activity increased throughout treatment, whereas NK cell activity was temporarily increased in treated compared with untreated mice but decreased to control levels before the end of treatment. Depletion of either CD8α or CD8β (data not shown) T cells in the mice eliminated the antitumor response, whereas depletion of NK1.1 had a modest effect on treatment outcome. Although the data do not indicate a role for CD4 cells in the curative effects seen, the flow cytometry data indicated that by day 5 these cells either had recovered significantly or may not have been completely depleted; therefore, a contribution of CD4 cells to therapeutic efficacy cannot be definitively excluded. Earlier studies (13, 14) in the EL4-C57BL/6 model had indicated that CD4+ cells are among the first to recover from this antibody treatment regimen, but in that study a 34% recovery was not seen until day 9 or 10 and a partial dependency on these cells for response was shown. As a whole, these data indicate that doxorubicin plus IL-2 treatment increased CTL activity and that CD8α+ T cells (classic CTL ref. 21) were required for complete response to treatment.

Splenocytes from untreated mice with advanced tumors had antitumor cytolytic activities that were somewhat lower than those of splenocytes from naive (i.e., no tumor, no treatment) mice. This suggested that E0771 tumors cause immune suppression. When splenocytes from E0771 mice were used, it showed that IL-2 treatment did not develop tumor when inoculated with E0771 cells but did develop tumor following inoculation of either EL4 lymphoma or Lewis lung carcinoma, tumors that are also syngeneic to C57BL/6 mice. These results show that the mice cured by the doxorubicin plus IL-2 treatment retained their ability to effectively and specifically recognize E0771 cancer cells after the first 20 days of treatment. In a subset of these mice, tumor regrowth occurred which indicated that viable E0771 tumor cells were still present. Because IL-2 alone had no effect on tumor growth in mice and because in culture IL-2 did not alter cell sensitivity to doxorubicin, it is unlikely that it had a direct effect on the tumor. This suggests that the prolonged IL-2 treatment in the combination therapy acts to decrease the percentage of mice that relapse rather than to increase the percentage of mice that respond. Over 80% of the mice cured of E0771 breast cancer by doxorubicin plus IL-2 treatment did not develop tumor when re inoculated with E0771 cells but did develop tumor following inoculation of either EL4 lymphoma or Lewis lung carcinoma, tumors that are also syngeneic to C57BL/6 mice. These results show that the mice cured by the doxorubicin plus IL-2 treatment retained their ability to effectively and specifically recognize E0771 cancer cells after treatment and strongly suggest that specific anti-E0771 immune memory had been established.

Figure 4. E0771 tumor induces immnosupression. Splenocytes (1 x 10⁴ or 1 x 10⁵) from naive mice (i.e., neither tumor-bearing nor treated), nonresponding treated E0771-bearing mice, or untreated E0771-bearing mice [potential reponders: CTL activity was generated and assayed as described for Fig. 3. **, P ≤ 0.003; ***, P ≤ 0.001; ***, P ≤ 0.0005, statistically significant changes in activity compared with effector cells alone. One of two representative experiments.

Figure 5. Effect of specific immune cell subset depletion on therapeutic efficacy of the doxorubicin + IL-2 combination treatment. The dependence of anti-E0771 tumor response on specific immune cell depletion. The dependence of anti-E0771 tumor response on specific immune cell depletion was tested by antibody-mediated specific immune cell depletion. Antibodies [16 μL anti-CD4 or anti-CD8β, antibody or 5 μL PK136 (anti-NK1.1) or Ly2.43 (anti-CD8α) hybridoma ascites fluid] were injected twice, 24 hours apart, before the mice (9-10 mice per treatment variable) were inoculated with the E0771 tumor cells (2.5 x 10⁵ per mouse). Antibodies were also given on days 10, 11, 20, 21, 30, and 31. For NK/CD8α and CD8α/NK switchover experiments, injections on days −2, −1, 10, and 11 were PK136 and Ly2.43, respectively, and injections on days 20, 21, 30, and 31 were the other antibody. Tumor size was measured every 2 to 3 days. All complete responder mice remained tumor free for the period they were monitored (at least 150 days). Except for the untreated control, all other groups were treated with doxorubicin + IL-2. Single experiment representative of multiple independent experiments (CD4, CD8α, and NK/CD8α switchover cell depletion experiments were done twice; CD8α and NK cell depletion experiments were done thrice).
metabonomic methodologies that sera from mice that had responded to treatment had a specific NMR spectral profile with high expression of chemicals in a cluster labeled factor 2. Data were also obtained that indicate that this NMR-based metabonomic assay may predict early during therapy which mice will respond to treatment. Preliminary analysis has identified three major metabolites among those that make up factor 2. Additional studies should further define which chemicals among those in the factor 2 cluster are the best predictors of response.

Our current hypothesis of the mechanism of action of the doxorubicin plus IL-2 combination treatment is that doxorubicin initially causes a modulation of the immunologic environment [e.g., thymus cellular makeup (22) and/or an appropriately timed initial immune response events. IL-2 is given clinically at very high doses for a short period of time (9, 12). Under these regimens, known for their toxicity, IL-2 is thought to provide all the stimulation for an anticancer immune response. In fact, the addition of cytotoxic chemotherapeutic drugs to high dose IL-2 regimens does not result in increased efficacy (12). Our nontoxic treatment, which uses prolonged administration of moderate doses of IL-2 in combination with a chemotherapeutic drug at a noncytotoxic immunomodulating dose, represents a new approach to cancer therapy that deserves clinical testing.

Acknowledgments

Received 11/10/2005; revised 2/22/2006; accepted 3/14/2006.

Grant support: Roswell Park Alliance Foundation (MJ, Ehrke and J. Alderfer), USPHS-NCI grant CA 15142, and USPHS-NCI core grant 16056.

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We thank Susan Staples for technical assistance.

References

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