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Gemcitabine Exerts a Selective Effect on the Humoral Immune Response: Implications for Combination Chemo-immunotherapy[1]

Anna K. Nowak, Bruce W. S. Robinson, and Richard A. Lake[2]

Western Australian Institute for Medical Research University Department of Medicine, Queen Elizabeth II Medical Centre, Perth, Western Australia 6009

ABSTRACT

Most cytotoxic drugs have gross effects on the immune system, such as neutropenia and lymphopenia. However, their effects on tumor-specific immune responses are unknown. Gemcitabine is a nucleoside analogue that is frequently used to treat non-small cell lung cancer. It is also active in other malignancies, either alone or in combination with cisplatin. Here, we investigate its effects on antigen-specific antitumor immunity using a murine tumor cell line transfected to express influenza virus hemagglutinin (HA). $CD4^+$, $CD8^+$, and $B220^+$ lymphocyte numbers all decreased during chemotherapy (120 μg/g, i.p., every third day for five doses), but B cells were selectively depleted. Gemcitabine induced a profound suppression of the IgG antibody response to HA, and this was unrelated to tumor size. In contrast, *in vitro* T-lymphocyte recall responses to the class I- and class II-restricted dominant peptide epitopes of HA were enhanced in tumor-bearing, gemcitabine-treated mice. We found that gemcitabine was >2-fold more potent in its ability to inhibit B-lymphocyte proliferation compared with T-lymphocyte proliferation. Thus, gemcitabine does not appear to be detrimental to specific antitumor cellular immunity and may be useful in combination chemo-immunotherapy protocols. In contrast, vaccination protocols requiring a humoral immune response for maximal efficacy may be compromised in patients treated with gemcitabine.

INTRODUCTION

Cytotoxic chemotherapy, surgery, and radiotherapy are the major modalities used in cancer treatment. Because cytotoxic drugs target dividing cells, the metabolic similarities between normal and neoplastic cells lead to low therapeutic indices and high toxicity. Bone marrow is particularly susceptible to cytotoxic damage, and the majority of cytotoxic drugs cause neutropenia and, to some degree, lymphopenia, thrombocytopenia, and anemia. Agents used to treat hematological malignancies in particular can cause severe lymphopenia and profound defects in cell-mediated immunity leading to opportunistic infections (1). Chemotherapy for childhood leukemia can lead to prolonged decreases in the numbers of T and B lymphocytes, with imbalance in T-lymphocyte subsets persisting for several months after treatment (2). What has been less clear is the effect of chemotherapy on antitumor immunity. Clearly, the destruction of tumor cells by cytotoxic drugs will increase the load of tumor antigen reaching the draining lymph node. The fact that this increased load fails to generate an immune response that controls minimal residual disease is assumed to be due to immune suppression. However, this has never been systematically evaluated.

Most tumors express an array of antigens that could act as targets for their immune-mediated destruction, and a number of potential therapies have emerged to exploit this (3). Immunotherapies, at least in animal models, have their most potent effects if they are administered when the tumor burden is small. Because cytotoxic drugs generate high rates of response in animals with established tumors, we reasoned that despite the *caveat* expressed above, combinations of chemotherapy and immunotherapy may act synergistically against established cancer.

Gemcitabine (2′,2′-difluorodeoxycytidine) is a nucleoside analogue of cytidine that is active as a single agent and in combination with cisplatin and other drugs against a wide range of solid tumors (4–7). It becomes incorporated into DNA with the subsequent addition of one further base to the DNA strand, a process known as "masked chain termination" (8). It thus halts DNA synthesis and is invisible to DNA repair systems, leading the cell into the apoptotic pathway. Furthermore, gemcitabine inhibits ribonucleotide reductase enzyme activity (9). Ribonucleotide reductase is a rate-limiting enzyme in DNA synthesis because it converts ribonucleotide diphosphates into deoxyribonucleotide diphosphates. Gemcitabine therefore decreases the deoxynucleotide triphosphate pool, causing a competitively higher incorporation of itself, as compared with dCTP, into the DNA. Although few data are available on the immunotoxicity of gemcitabine, other nucleoside analogues such as fludarabine cause major alterations in the CD4:CD8 lymphocyte ratios, associated with marked increases in the rate of opportunistic infections (1).

In this study, we show that gemcitabine massively depletes lymphocyte numbers, but it has a selective detrimental effect on the B-lymphocyte subset, associated with a complete ablation of the antitumor antibody response. In contrast, tumor-specific $CD4^+$ and $CD8^+$ recall responses were augmented. These observations are at least partially explained by the *in vitro* finding that B lymphocytes are more sensitive to the antiproliferative effects of gemcitabine than T lymphocytes. Taken together, the data anticipate successful combinations of gemcitabine with immunotherapy protocols in the treatment of cancer.

MATERIALS AND METHODS

Mice. BALB/C (H-2^d) mice were obtained from the Animal Resources Center (Perth, Australia) and maintained under standard conditions in the University Department of Medicine animal holding area. Two lines of anti-HA[3] TCR transgenic mice were used. Clone 4 TCR transgenic mice (CL4; Ref. 10) express a TCR recognizing the dominant class I-restricted HA epitope and were backcrossed with BALB/C mice for at least eight generations. HNT TCR transgenic mice were also backcrossed with BALB/C mice for at least eight generations. These animals express a class II-restricted receptor that recognizes the dominant class II-restricted HA epitope (11). Animals were bred in the university specific pathogen-free animal facility, and experimental animals were maintained in a non-specific pathogen-free facility. All mice used in these studies were between 8 and 12 weeks of age.

Cell Lines. All cell lines were regularly tested and remained negative for *Mycoplasma* spp. The AB1 murine malignant mesothelioma cell line was generated by injecting crocidolite asbestos i.p. into BALB/C mice, and the peritoneal exudate was passaged *in vitro* and *in vivo* until stable clonal cell lines were obtained (12). Cell lines were maintained in RPMI 1640 (Invitrogen) supplemented with 20 mM HEPES, 0.05 mM 2-mercaptoethanol, 100 units/ml penicillin (CSL), 50 μg/ml gentamicin (David Bull Laboratories), and

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[2] To whom requests for reprints should be addressed, at University Department of Medicine, Queen Elizabeth II Medical Centre, 4th Floor, G Block, Nedlands, Perth, Western Australia 6009. Phone: 618-9346-3127; Fax: 618-9346-2816; E-mail: rlake@cyllene.uwa.edu.au.

[3] The abbreviations used are: HA, hemagglutinin; TCR, T-cell receptor; NK, natural killer; LAK, lymphokine-activated killer.

5% FCS (Invitrogen). AB1 is a class I^+, class II^- tumorigenic cell line. AB1 cells were transfected with the murine influenza HA gene (AB1-HA; Ref. 13). Transfected cells were maintained in media containing the neomycin analogue Geneticin (Invitrogen) at a final concentration of 400 μg/ml. Expression of HA was measured by fluorescence-activated cell-sorting analysis before use in each experiment. Gemcitabine-resistant AB1-HA (AB1-HA-GR250) was generated by culturing cells in media containing progressively increasing concentrations of the drug (Eli Lilly). At each concentration, the cell line was passaged until growth rates were the same as that of the untreated parent cell line before increasing the concentration of gemcitabine. This cell line was grown and passaged in a final concentration of 1.67 μg/ml gemcitabine. The IC_{50}, as assessed by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, was >800-fold that of the parent cell line. AB1-HA-GR250 maintained equivalent expression of the HA antigen.

Experimental Protocol. AB1-HA tumor cells (1×10^6) were inoculated s.c. on one side of the ventral surface in the lower flank region. In general, standard chemotherapy commenced 9 days later, when a small palpable lump (ranging from 1–2 mm in diameter) was evident. Mice were injected i.p. with 120 μg/g gemcitabine every third day for five doses, a regimen previously established as the maximal tolerated dose for BALB/C mice (14). Control mice received PBS vehicle alone. Mice were weighed before each dose, and the dose was adjusted for individual mice. Tumor size was measured with callipers three times weekly during the course of chemotherapy and subsequently measured until tumor size reached approximately 10×10 mm, at which point mice were culled. In some experiments, single cell suspensions of TCR transgenic lymphocytes were donated by i.v. injection. Adoptive transfer consisted of 2×10^7 cells comprising equal numbers of HNT and CL4 lymphocytes in a total volume of 200 μl.

Anti-influenza Virus Antibody ELISA. PR8 virus (2500 units/ml) was prepared and purified as described previously (13). Virus was diluted in coating buffer and aliquoted into 96-well flexible microtiter plates. After overnight incubation, plates were washed, and unreacted sites were blocked by adding 200 μl of PBS and 10% horse serum to all wells. Blanks, test, and control sera and standards were added to test wells. The standard used was H-18 anti-HA antibody. This was followed by sequential incubations using biotinylated sheep antimouse IgG (Jackson ImmunoResearch Laboratories), streptavidin-conjugated horseradish peroxidase (Dako), and the horseradish peroxidase substrate 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma-Aldrich). Serum antibody levels were interpolated from absorbance data determined using a SpectraMax 250 spectrophotometer at $A_{405\ nm}$ with a reference of 490 nm (Molecular Devices).

Lymphocyte Proliferation Assays. Single cell suspensions of lymphocytes were washed and resuspended in RPMI 1640 with 5% FCS at 2×10^5 cells/well in 50 μl and seeded into 96-well flat-bottomed tissue culture plates (Falcon). Some wells were precoated with either 200 μl of antimurine CD3 (monoclonal antibody KT3.2; American Type Culture Collection; 1 mg/ml in PBS) or antimurine IgM antibody (Silenus) overnight at 4°C; otherwise, mitogens were added to the cultures [lipopolysaccharide (Sigma-Aldrich; 1 μg/ml); peptides (Chiron Technologies) were serially diluted from 10 μg/ml]. Anti-CD40-driven proliferation was stimulated by FGK45 antibody (a gift of Dr. Antonius Rolink, Basel Institute for Immunology) at a concentration of 10 μg/ml. For experiments to determine the IC_{50} of gemcitabine on stimulated lymphocyte populations, serial dilutions of gemcitabine were added to the cultures. After a 48-h incubation, wells were pulsed with [^{3}H]thymidine (1 μCi/well) for 15 h and harvested onto filter paper for scintillation counting. All assays were done in triplicate.

Statistical Analysis. Data comparing differences between groups were assessed using Student's *t* test and/or the Mann-Whitney nonparametric *U* test. Differences were considered significant when *P* was <0.05. Statistical analysis was conducted using the SPSS for Windows program.

RESULTS

Gemcitabine Inhibits Tumor Growth and Leads to Increased Survival. Mice were given standard therapy [gemcitabine (120 μg/g) or vehicle, i.p., every third day for five doses] 9 days after tumor inoculation. Tumor sizes ranged from just palpable to 2×2 mm. Mice treated with gemcitabine showed significant growth delay of tumors compared with mice treated with vehicle (Fig. 1*a*). Standard therapy was then initiated when the mean tumor size was approximately 50 mm^2 (19 days after inoculation). All mice in the control group were culled 6 days later because of large tumor size (>100 mm^2). Mice treated with gemcitabine showed a decrease in mean tumor size of >50% during the treatment period (Fig. 1*b*) and exhibited prolonged survival. Gemcitabine is therefore an effective treatment in this system.

Gemcitabine Causes Splenic Involution Associated with Decreased Cellularity. We evaluated changes in total cell numbers in lymph nodes and spleens of tumor-bearing mice undergoing standard therapy and compared these with normal values. Tumor-bearing mice receiving vehicle therapy had fewer cells in the spleen than did normal mice, but the differences were not significant. However, total spleen cell numbers were significantly lower in gemcitabine-treated tumor-bearing mice than in control tumor-bearing mice throughout treatment (Fig. 2). At the nadir, splenocyte numbers were <15% of normal. Spleen cellularity recovered by day +21 (9 days after the final injection of gemcitabine), and there was an indication of rebound hypercellularity. There were no significant differences in lymph node cellularity between the groups (data not shown). Although there was a trend toward lower cell numbers during gemcitabine treatment, there was more variability in lymph node cellularity than spleen cellularity.

Gemcitabine Differentially Targets Lymphocyte Subsets in the Spleen. Cells obtained from animals treated with gemcitabine as described above were stained and analyzed by flow cytometry for the lymphocyte markers CD4, CD8, and B220 (Fig. 3). $CD8^+$ cell counts in the spleen were lower at days +1 and +5 ($P < 0.05$), but not at day +13 or day +21. $CD4^+$ cell counts were lower at days +1 ($P < 0.08$), +5 ($P < 0.01$), and +13 ($P < 0.001$), but not at day +21. Similarly, $B220^+$ cell counts were lower at days +1 ($P < 0.001$), +5 ($P < 0.005$), and +13 ($P < 0.004$) but had recovered to normal by day +21. These results mirror the decrease in total cell numbers seen

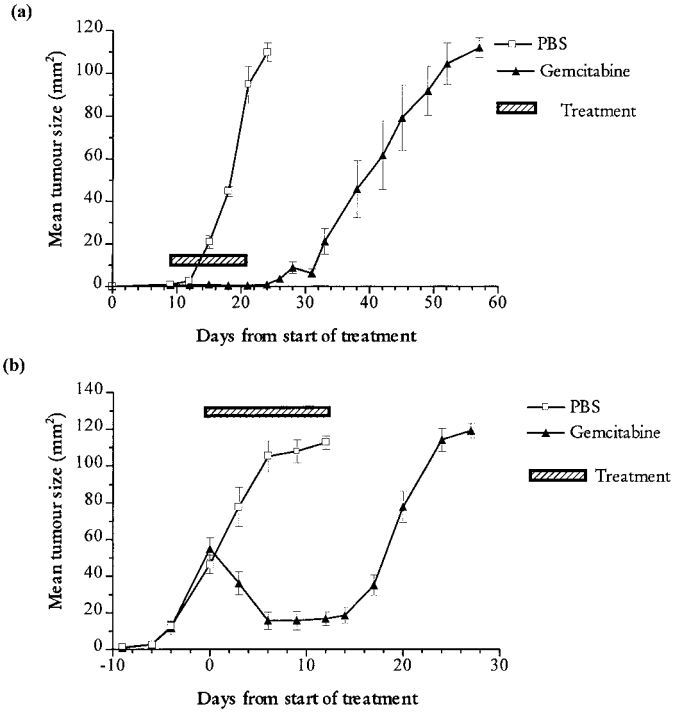

Fig. 1. Mice (5 mice/group) with established AB1-HA tumors were treated with five doses of gemcitabine or PBS injection at 3-day intervals from 9 days after tumor inoculation (*a*) or from the time at which tumors reached a mean size of 50 mm^2 (*b*). Tumor size was calculated as the product of two measurements obtained using calipers. This experiment was repeated on three occasions with similar results.

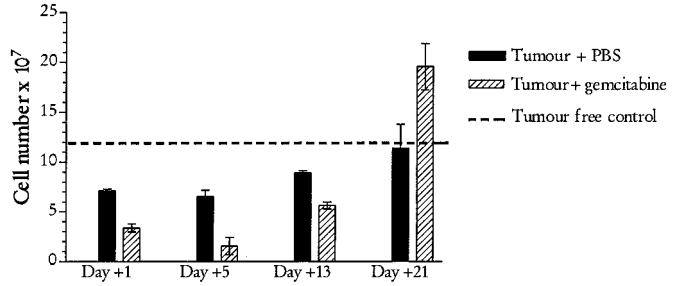

Fig. 2. Mice (3 mice/group) were treated as described in Fig. 1 from 9 days after tumor inoculation. Groups were culled at 1, 5, 13, or 21 days after the start of treatment, and spleen cellularity was assessed by cell counting. Statistically significant differences were observed between the groups at day +1 ($P < 0.04$), day +5 ($P < 0.01$), and day +13 ($P < 0.01$).

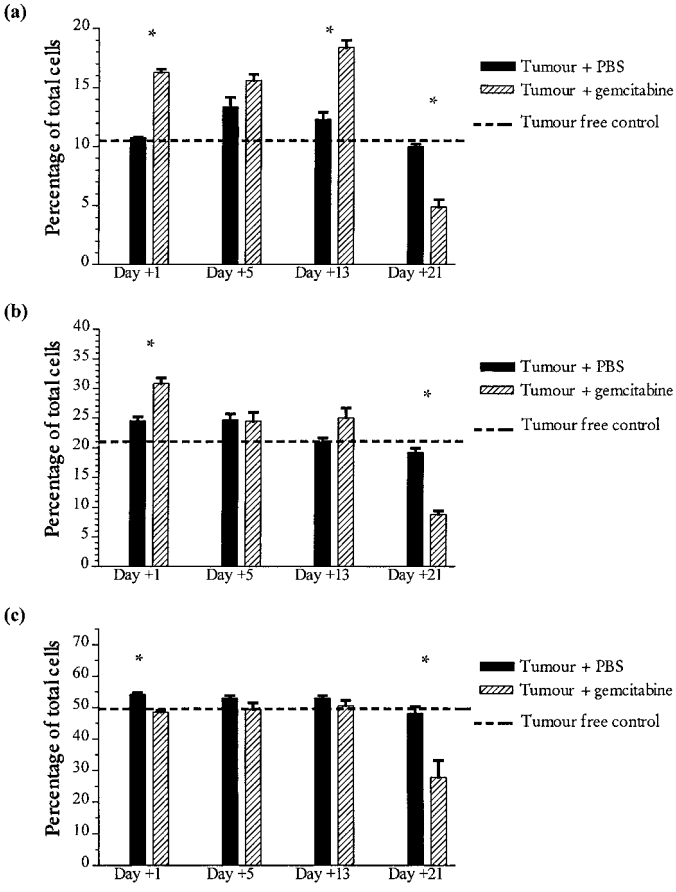

Fig. 3. Mice (3 mice/group) were treated as described in Fig. 2. Groups were culled at 1, 5, 13, or 21 days after the start of treatment, and spleen cell subsets (*a*, CD8; *b*, CD4; *c*, B cells) were counted by fluorescence-activated cell-sorting analysis. Statistically significant differences between the groups are marked with an *asterisk* ($P < 0.05$).

at days +1, +5, and +13. As a percentage of total spleen cells, $CD8^+$ and $CD4^+$ T cells were significantly increased at day +1 ($P < 0.01$), and $CD8^+$ T cells were also significantly increased at day +13 ($P < 0.002$). In contrast to the overall increase in the proportion of T cells throughout gemcitabine treatment, B-cell proportions were generally lower and significantly decreased at day +1 ($P < 0.003$). All subsets were proportionally decreased at day +21 ($P < 0.03$), most likely due to the infiltration of immature hematopoietic cells associated with bone marrow recovery. Overall, the profound decrease in spleen cellularity occurring with gemcitabine treatment is explained by a partial loss in the T-lymphocyte compartment and a correspondingly greater decrease in absolute numbers of B cells.

Gemcitabine Abrogates the Induction of Antitumor Antibodies. Serum was collected at days 1, 5, 13, and 21 after the start of standard therapy. Anti-influenza virus IgG production was assessed by ELISA. Antibody titers increased with increasing tumor burden in the control mice. In contrast, antibody titers were markedly suppressed in gemcitabine-treated mice from day 13 ($P < 0.05$). Titers remained low 9 days after the cessation of chemotherapy ($P < 0.05$) despite tumor regrowth (Fig. 4).

Gemcitabine Abrogates Antibody Induction Independently of Tumor Presence. Because tumors continue to grow when they are untreated, the antigen load increases correspondingly, and this has been shown to affect the magnitude of antigen presentation in the draining lymph node (15). Gemcitabine could therefore abrogate the antibody response to tumor as a direct consequence of its effect on limiting tumor growth. Gemcitabine has the potential to inhibit proliferation of live virus (16), so we used a strategy of vaccination with heat-killed virus to ensure that the same antigen load was encountered in both groups of mice. Vaccinated animals received two doses of gemcitabine, and serum was obtained as above. Gemcitabine treatment significantly inhibited anti-influenza virus antibody formation when compared with treatment with vehicle alone ($P < 0.0001$; Fig. 5). These results indicate that the suppressive effect on specific antitumor antibody production observed above is independent of antigen load.

Gemcitabine Abrogates the Induction of Antitumor Antibodies in Mice Bearing Gemcitabine-resistant Tumors. To determine whether factors elicited by growing tumors could affect the humoral immune response and to confirm that any effects were not secondary to tumor shrinkage, we repeated the experiment described above with

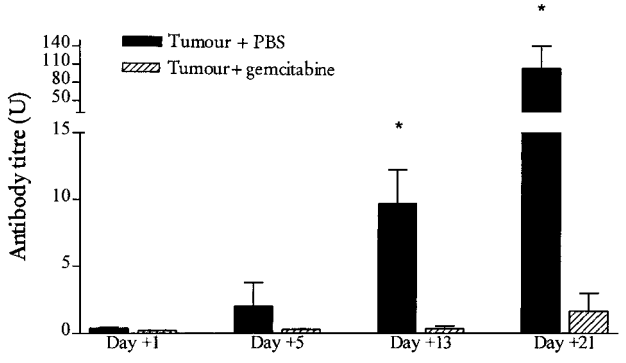

Fig. 4. Mice (5 mice/group) were treated as described in Fig. 1. Sera were obtained at days 1, 5, 13, and 21 after the start of treatment, and anti-influenza virus antibody titer (IgG) was assessed by ELISA. Statistically significant differences between the groups are marked with an *asterisk* ($P < 0.05$). This experiment was repeated on three occasions with similar results.

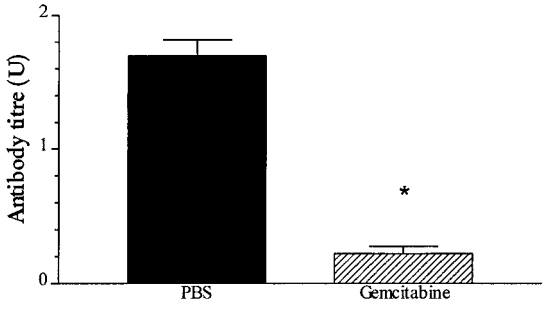

Fig. 5. Mice (5 mice/group) were inoculated with heat-killed PR8 virus. After two doses of gemcitabine or PBS control treatment, sera were tested for anti-influenza virus antibodies by ELISA. Mice treated with gemcitabine showed profound suppression of anti-HA IgG despite identical antigen load. *, $P < 0.0001$ This experiment was repeated on three occasions with similar results.

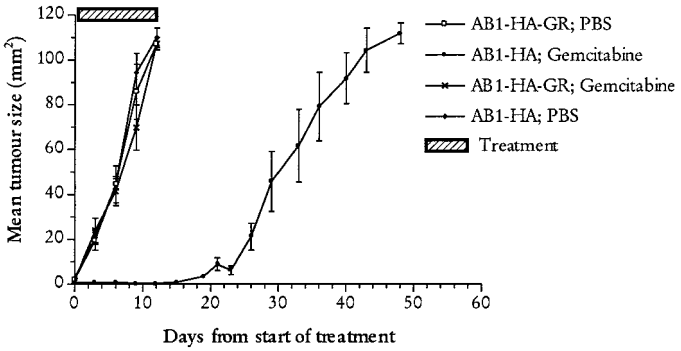

Fig. 6. Four groups of mice (5 mice/group) were inoculated with a gemcitabine-resistant cell line, AB1-HA-GR250, or with AB1-HA. Groups were then treated with gemcitabine or control standard therapy as described in Fig. 1.

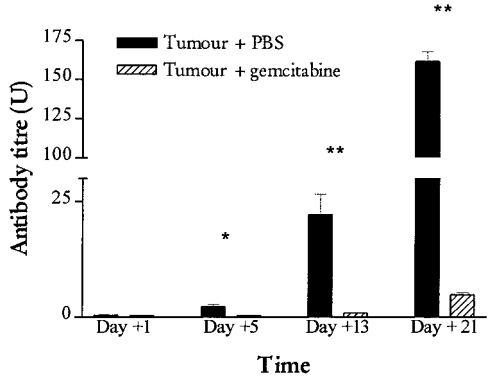

Fig. 7. Mice (5 mice/group) were inoculated with the gemcitabine-resistant cell line AB1-HA-GR250. Standard therapy commenced 9 days later. Sera were obtained at days 1, 5, 13, and 21 after the start of treatment and tested for anti-influenza virus antibodies by ELISA. *, $P < 0.06$; **, $P < 0.001$. This experiment was repeated with similar results.

a gemcitabine-resistant cell line (AB1-HA-GR250). The rate of tumor growth was identical in mice treated with gemcitabine or vehicle and did not differ from the growth rate of the untreated parent cell line (AB1-HA; Fig. 6). As with the drug-sensitive tumor, gemcitabine inhibited antibody formation (Fig. 7).

Tumor Antigen-specific Cellular Responses Are Enhanced by Gemcitabine. Cellular proliferation was measured *in vitro* at 1, 5, 13, and 21 days after the start of standard therapy. To increase the frequency of antigen-specific precursors, mice were given tumor antigen-specific CD4 (HNT) and CD8 (CL4) lymphocytes by adoptive transfer 1 day before the start of treatment. Gemcitabine-treated animals exhibited decreased proliferation to both polyclonal T-cell mitogens (anti-CD3; $P < 0.001$) and polyclonal B-cell mitogens (anti-IgM; $P < 0.01$). However antigen-specific proliferation was augmented (HNT, $P < 0.03$; CL4, $P < 0.002$), and this was apparent at all concentrations of peptide tested. A representative time point 5 days after the start of standard therapy is shown (Fig. 8), although the results were similar at all four time points studied and when the experiment was repeated three times.

Gemcitabine Selectively Suppresses B Lymphocyte Proliferation. To further investigate the selective effect of gemcitabine on cellular and humoral immune responses, the IC_{50} was determined for different cell types. The IC_{50} of gemcitabine for B cells stimulated by lipopolysaccharide was less than half that for T cells stimulated by anti-CD3 or for unselected splenocytes stimulated by the combination of phorbol 12-myristate 13-acetate and ionomycin (Fig. 9). The IC_{50} for T cells was similar to the IC_{50} for the tumor cells. Thus, at drug concentrations that are inhibitory to tumor growth, there is likely to be a more profound suppressive effect on B-cell activity.

DISCUSSION

Many clinical trials have shown that combinations can be more effective than the single use of any particular modality. However, there have been few studies combining immunotherapy and chemotherapy in cancer, mostly because it has been widely assumed that chemotherapy is immunosuppressive and would negate the benefits of immunotherapy. We reasoned that this might not be the case if chemotherapy exposed the host immune system to large quantities of tumor antigen. This study therefore provides a foundation for a rational approach to understanding changes in antigen-specific antitumor immunity induced by chemotherapy to enable the effective use of adjunct immunotherapy.

This study shows that gemcitabine causes a profound depletion of lymphocytes. It is selectively detrimental to the humoral immune response but spares antigen-specific cellular immunity. Our finding that antigen-specific B-lymphocyte immunity is severely impaired by gemcitabine does not have obvious negative implications for antitumor immunity because the crucial role of $CD4^+$ and $CD8^+$ T cells in antitumor immunity is now widely acknowledged. Adoptive transfer studies using $CD8^+$ T-cell lines and $CD8^+$ clones specific for tumor antigens have demonstrated that these cells are the major effectors of the antitumor response (17). Similarly, $CD4^+$ T cells have been shown to be important participants in an effective antitumor response in their role providing T-cell help (13). In humans, the important role of T lymphocytes in antitumor immunity is demonstrated by the presence of graft-*versus*-malignancy effects after allogeneic bone

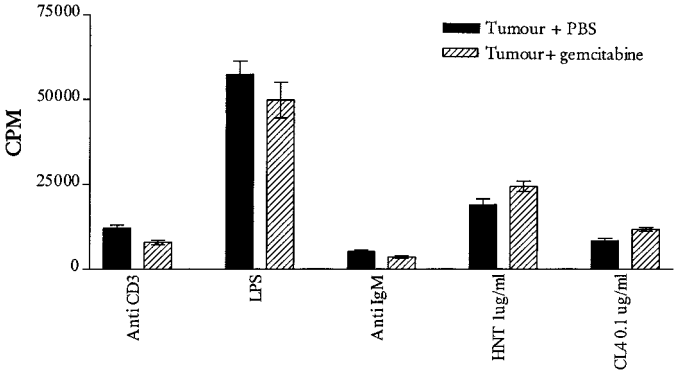

Fig. 8. Mice (4 mice/group) were treated as described in Fig. 1. Five days after commencement of standard therapy, lymph nodes were removed and disaggregated into single cell suspensions. Lymphocytes were exposed to different mitogens, and their proliferative response was assessed by [^{3}H]thymidine incorporation 48 h later. Results shown are the mean of triplicate wells from a single experiment. Statistically significant differences between the groups are recorded in the results. The experiment was repeated three times at 1, 5, 13, or 21 days after the start of treatment with similar results.

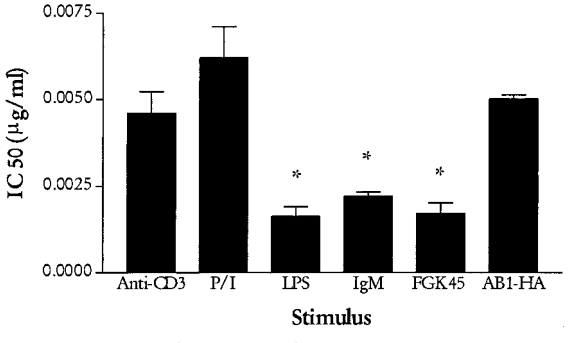

Fig. 9. Single cell suspensions of lymphocytes from non-tumor-bearing mice were stimulated with different mitogens *in vitro*. The cells were then incubated with varying concentrations of gemcitabine for 48 h, and the IC_{50} of gemcitabine was determined. Each point was assessed in triplicate, and data are the mean of three different experiments.

marrow transplantation. T-cell-depleted allotransplants are associated with an increased risk of relapse in patients with chronic myelogenous leukemia and acute myelogenous leukemia (18), and relapses can be treated by infusions of donor lymphocytes containing T cells (19). No such role has been demonstrated for B lymphocytes or antitumor antibody production in murine models or in humans.

Although antibody responses do not generally correlate with resistance to tumor, in some experimental situations, immune sera have been shown to enhance tumor growth, ostensibly by blocking access of tumor-specific lymphocytes to their target (20). The possibility that B cells and their products might inhibit the induction of T-cell-dependent tumor immunity has been more recently revisited by analyzing tumor immunity in B-cell-deficient mice (21). These mice controlled tumor growth more readily than their normal littermates, allowing the authors to conclude that the low immunogenicity of tumors is compounded by B cells, whose presence in the priming phase results in disabled $CD4^+$ T-cell help for $CD8^+$ CTLs.

Previous studies of immunotoxicity of chemotherapeutic agents have not reported any selective B-lymphocyte toxicity. Most studies in patient populations have been performed in groups receiving combination chemotherapy, and thus it is difficult to separate the effects of any one agent. Children and young adults undergoing intensive chemotherapy for solid tumors and lymphomas have been studied for changes in peripheral blood lymphocyte populations following maximal hematological recovery after several chemotherapy cycles. It was reported that lymphocyte recovery did not occur between cycles in these patients, leading to severe B- and T-cell depletion (22). B cells were nearly undetectable, and IgM was absent in 50% of the patients studied. IgG levels were normal. There was a progressive decrease in CD4 and CD8 lymphocyte numbers, with CD4 cells showing a disproportionate decrease that persisted for at least 4 months after chemotherapy in all patients studied. This group also studied patients with advanced breast cancer undergoing intensive chemotherapy with 5-fluorouracil, leucovorin, doxorubicin, cyclophosphamide, and paclitaxel. All patients showed a dramatic decrease in naïve CD4 T-cell populations, and increased susceptibility to apoptosis was observed in CD4 cells stimulated by mitogens after chemotherapy (23). The authors also hypothesize that this phenomenon could lead to T-cell decline rather than expansion when environmental pathogens or tumor antigens were encountered. Thus, attempts at immunotherapy in this situation may result in deletion from the T-cell repertoire of the tumor-specific T cells that were targeted for activation and expansion.

Breast cancer patients receiving adjuvant treatment with cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) have been investigated for lymphocyte proliferation to autologous tumor-associated antigens and other mitogens. The majority of patients had lower responses to both pokeweed mitogen (B lymphocytes) and phytohemagglutinin (T lymphocytes) after six cycles of chemotherapy. However, 50% of the patients tested showed an increase in autologous tumor antigen reactivity after chemotherapy (24). Another group studying the same chemotherapy regimen showed preservation of CD8 cell numbers, with a decrease in CD4 cell numbers over successive cycles, and a rapid decrease in B-cell numbers (25).

A study investigating the effects of gemcitabine in 16 patients with solid tumors showed no decrease in total peripheral blood lymphocyte numbers after gemcitabine therapy, with T cells increasing from 68% to 77% of total lymphocytes, NK cells decreasing from 15% to 10% of total lymphocytes, and the CD4:CD8 ratio increasing from 1.7 to 2.2. B-cell percentages were unchanged (26). The effect of gemcitabine on human hematopoietic progenitor cells has also been investigated using clonogenic assays of colony-forming units, granulocyte macrophage and burst-forming units, erythroid (27). They have shown potent inhibition of both progenitor lineages with continuous exposure to gemcitabine in a 13-day culture; however, this was 1000-fold less with a 1-h exposure to gemcitabine, an exposure that is consistent with its clinical use. It is doubtful whether the results of a 13-day culture with gemcitabine are relevant to the clinical situation. Of four patients undergoing a standard gemcitabine chemotherapy regimen, only one patient showing marked and sustained decreases in the levels of hematopoietic progenitors. In the other cases, short-lived decreases in peripheral blood progenitor levels alternated with dramatic increases. The lack of significant neutropenia seen clinically in patients undergoing chemotherapy with single agent gemcitabine is in keeping with these results. A study of the effects of gemcitabine treatment *in vitro* on NK cells, LAK cells, and alloreactive CTL activity showed marked inhibition of LAK cell generation with a smaller decrease in lytic efficiency of mature LAK cells (28). CTL generation was also strongly inhibited, as was the lytic response of mature CTLs, after a long coculture with gemcitabine. There were minimal effects on NK cell activity.

With the exception of Head *et al.* (24), these studies have not been able to assess the effects of chemotherapy on antigen-specific antitumor response. As our results demonstrate, changes in absolute nonspecific T- and B-cell numbers do not necessarily reflect the effects of treatment on the lymphocyte population with antigen specificity. Despite complete recovery of total B-cell numbers at 21 days after initiation of gemcitabine treatment, a profound defect in antitumor antibody production persisted. Similarly, $CD8^+$ and $CD4^+$ T-cell numbers were at their nadir at day 5, whereas, at the same time, recall responses to tumor antigens were enhanced. Thus, it cannot be assumed that low absolute lymphocyte numbers represent impaired antitumor immunity.

Whereas our findings do not support any detrimental effect of gemcitabine on antitumor immunity, the impairment of antibody formation suggests that vaccination protocols against specific pathogens that require a humoral response for maximal efficacy may lead to suboptimal disease protection. The majority of studies of response to vaccination in cancer patients have been performed in pediatric populations, and responses have been assessed after a variety of combination chemotherapy protocols. It is not possible to assess the contribution of any single cytotoxic agent. Nevertheless, humoral responses to diphtheria and tetanus toxin and poliomyelitis virus were depressed in members of a population that had undergone an adequate age-appropriate immunization protocol, many of whom had received cytotoxic chemotherapy (29). Serological responses to hepatitis B vaccine have also been shown to be markedly depressed in pediatric populations receiving combination chemotherapy (30, 31). In the adult population, vaccination against influenza virus is now widely recommended in patients with concomitant medical illness such as malignancy. The humoral immune response to influenza vaccine has been studied in a small group of nine women with breast cancer and did not differ significantly from the response of a control group of 19 healthy women (32). These studies have not been performed in groups of patients likely to be receiving treatment with gemcitabine, such as those with pancreatic or non-small-cell lung cancer.

Although this is a murine tumor model, it may be analogous to the use of chemotherapy in many clinical situations where the drug is effective in reducing tumor size and resulting in a partial response but rarely eradicates the tumor completely. In advanced disease, this is an ideal situation for the use of combination chemo-immunotherapy because the reduction in tumor bulk may allow immunotherapeutic strategies to overcome the challenge of entry into the tumor mass. Because gemcitabine has not demonstrated any detrimental effects on antitumor immunity, it may be an ideal drug to combine with immunotherapy strategies. This approach is currently being pursued in our animal model.

REFERENCES

1. Wijermans, P. W., Gerrits, W. B., and Haak, H. L. Severe immunodeficiency in patients treated with fludarabine monophosphate. Eur. J. Haematol., *50:* 292–296, 1993.
2. Mackall, C. L., Fleisher, T. A., Brown, M. R., Andrich, M. P., Chen, C. C., Feuerstein, I. M., Magrath, I. T., Wexler, L. H., Dimitrov, D. S., and Gress, R. E. Distinctions between CD8+ and CD4+ T-cell regenerative pathways result in prolonged T-cell subset imbalance after intensive chemotherapy. Blood, *89:* 3700–3707, 1997.
3. Melief, C. J., Toes, R. E., Medema, J. P., van der Burg, S. H., Ossendorp, F., and Offringa, R. Strategies for immunotherapy of cancer. Adv. Immunol., *75:* 235–282, 2000.
4. Manegold, C., Zatloukal, P., Krejcy, K., and Blatter, J. Gemcitabine in non-small cell lung cancer (NSCLC). Investig. New Drugs, *18:* 29–42, 2000.
5. Oettle, H., Arnold, D., Hempel, C., and Riess, H. The role of gemcitabine alone and in combination in the treatment of pancreatic cancer. Anticancer Drugs, *11:* 771–786, 2000.
6. Orlando, M., and Mandachain, M. Gemcitabine in ovarian cancer. Semin. Oncol., *28:* 62–69, 2001.
7. von der Maase, H. Gemcitabine-containing regimens in bladder cancer: a new standard of care. Semin. Oncol., *28:* 1–3, 2001.
8. Storniolo, A. M., Allerheiligen, S. R., and Pearce, H. L. Preclinical, pharmacologic, and Phase I studies of gemcitabine. Semin. Oncol., *24:* S7-2−S7-7, 1997.
9. Goan, Y. G., Zhou, B., Hu, E., Mi, S., and Yen, Y. Overexpression of ribonucleotide reductase as a mechanism of resistance to 2,2-difluorodeoxycytidine in the human KB cancer cell line. Cancer Res., *59:* 4204–4207, 1999.
10. Morgan, D. J., Liblau, R., Scott, B., Fleck, S., McDevitt, H. O., Sarvetnick, N., Lo, D., and Sherman, L. A. CD8(+) T cell-mediated spontaneous diabetes in neonatal mice. J. Immunol., *157:* 978–983, 1996.
11. Scott, B., Liblau, R., Degermann, S., Marconi, L. A., Ogata, L., Caton, A. J., McDevitt, H. O., and Lo, D. A role for non-MHC genetic polymorphism in susceptibility to spontaneous autoimmunity. Immunity, *1:* 73–82, 1994.
12. Davis, M. R., Manning, L. S., Whitaker, D., Garlepp, M. J., and Robinson, B. W. Establishment of a murine model of malignant mesothelioma. Int. J. Cancer, *52:* 881–886, 1992.
13. Marzo, A. L., Lake, R. A., Robinson, B. W., and Scott, B. T-cell receptor transgenic analysis of tumor-specific CD8 and CD4 responses in the eradication of solid tumors. Cancer Res., *59:* 1071–1079, 1999.
14. Braakhuis, B. J. M., Vanhaperen, V., Boven, E., Veerman, G., and Peters, G. J. Schedule-dependent antitumor effect of gemcitabine in *in vivo* model systems. Semin. Oncol., *22:* 42–46, 1995.
15. Marzo, A. L., Lake, R. A., Lo, D., Sherman, L., McWilliam, A., Nelson, D., Robinson, B. W. S., and Scott, B. Tumor antigens are constitutively presented in the draining lymph nodes. J. Immunol., *162:* 5838–5845, 1999.
16. Plunkett, W., Huang, P., Searcy, C. E., and Gandhi, V. Gemcitabine: preclinical pharmacology and mechanisms of action. Semin. Oncol., *23:* 3–15, 1996.
17. Melief, C. J. Tumor eradication by adoptive transfer of cytotoxic T lymphocytes. Adv. Cancer Res., *58:* 143–175, 1992.
18. Horowitz, M. M., Gale, R. P., Sondel, P. M., Goldman, J. M., Kersey, J., Kolb, H. J., Rimm, A. A., Ringden, O., Rozman, C., Speck, B., *et al.* Graft-*versus*-leukemia reactions after bone marrow transplantation. Blood, *75:* 555–562, 1990.
19. Collins, R. H., Jr., Shpilberg, O., Drobyski, W. R., Porter, D. L., Giralt, S., Champlin, R., Goodman, S. A., Wolff, S. N., Hu, W., Verfaillie, C., List, A., Dalton, W., Ognoskie, N., Chetrit, A., Antin, J. H., and Nemunaitis, J. Donor leukocyte infusions in 140 patients with relapsed malignancy after allogeneic bone marrow transplantation. J. Clin. Oncol., *15:* 433–444, 1997.
20. Hellstrom, K. E., and Hellstrom, I. Lymphocyte-mediated cytotoxicity and blocking serum activity to tumor antigens. Adv. Immunol., *18:* 209–277, 1974.
21. Qin, Z., Richter, G., Schuler, T., Ibe, S., Cao, X., and Blankenstein, T. B cells inhibit induction of T cell-dependent tumor immunity. Nat. Med., *4:* 627–630, 1998.
22. Mackall, C. L., Fleisher, T. A., Brown, M. R., Magrath, I. T., Shad, A. T., Horowitz, M. E., Wexler, L. H., Adde, M. A., McClure, L. L., and Gress, R. E. Lymphocyte depletion during treatment with intensive chemotherapy for cancer. Blood, *84:* 2221–2228, 1994.
23. Hakim, F. T., Cepeda, R., Kaimei, S., Mackall, C. L., McAtee, N., Zujewski, J., Cowan, K., and Gress, R. E. Constraints on CD4 recovery postchemotherapy in adults: thymic insufficiency and apoptotic decline of expanded peripheral CD4 cells. Blood, *90:* 3789–3798, 1997.
24. Head, J. F., Elliott, R. L., and McCoy, J. L. Evaluation of lymphocyte immunity in breast cancer patients. Breast Cancer Res. Treat., *26:* 77–88, 1993.
25. Sewell, H. F., Halbert, C. F., Robins, R. A., Galvin, A., Chan, S., and Blamey, R. W. Chemotherapy-induced differential changes in lymphocyte subsets and natural-killer-cell function in patients with advanced breast cancer. Int. J. Cancer, *55:* 735–738, 1993.
26. Daikeler, T., Maas, K., Weiss, B., Hartmann, J. T., Knobloch, A., Arning, M., Kanz, L., and Bokemeyer, C. The influence of gemcitabine on the CD4/CD8 ratio in patients with solid tumours. Oncol. Rep., *4:* 561–564, 1997.
27. Botta, M., Castello, G., Ballarino, P., Arboscello, E., Lerza, R., Mencoboni, M., and Pannacciulli, I. Toxicity on human hemopoietic progenitors of 2′-2′-difluoro-2′deoxycytidine (gemcitabine). Anticancer Res., *18:* 1037–1042, 1998.
28. Alvino, E., Fuggetta, M. P., Tricarico, M., and Bonmassar, E. 2′-2′-Difluorodeoxycytidine: *in vitro* effects on cell-mediated immune response. Anticancer Res., *18:* 3597–3602, 1998.
29. von der Hardt, K., Jungert, J., Beck, J. D., and Heininger, U. Humoral immunity against diphtheria, tetanus and poliomyelitis after antineoplastic therapy in children and adolescents: a retrospective analysis. Vaccine, *18:* 2999–3004, 2000.
30. Hovi, L., Valle, M., Siimes, M. A., Jalanko, H., and Saarinen, U. M. Impaired response to hepatitis B vaccine in children receiving anticancer chemotherapy. Pediatr. Infect. Dis. J., *14:* 931–935, 1995.
31. Polychronopoulou-Androulakaki, S., Panagiotou, J. P., Kostaridou, S., Kyratzopoulou, A., and Haidas, S. Immune response of immunocompromised children with malignancies to a recombinant hepatitis B vaccine. Pediatr. Hematol. Oncol., *13:* 425–431, 1996.
32. Brydak, L. B., Guzy, J., Starzyk, J., Machala, M., and Gozdz, S. S. Humoral immune response after vaccination against influenza in patients with breast cancer. Support Care Cancer, *9:* 65–68, 2001.

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Anna K. Nowak, Bruce W. S. Robinson and Richard A. Lake

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