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

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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<sup>+</sup>, CD8<sup>+</sup>, and B220<sup>+</sup> 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 adminis-

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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-GR250 (AB1-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-GR250 maintained equivalent expression of the HA antigen.

**Experimental Protocol.** AB1-GR250 tumour cells (1 x 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 x 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 x 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 x 10^5 cells/well in 50 μl. Lymphocyte pellets were placed on a 21-day growth curve in RPMI 1640. Cells were then transferred to a 96-well plate. AB1-HA tumors were established as the maximal tolerated dose for BALB/C mice (14). Control 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 in tumors compared with mice treated with vehicle (Fig. 1a). 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. 1b) and exhibited prolonged survival. Gemcitabine is therefore an effective treatment in this system.

**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 x 2 mm. Mice treated with gemcitabine showed significant growth delay of tumors compared with mice treated with vehicle (Fig. 1a). 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. 1b) and exhibited prolonged survival. Gemcitabine is therefore an effective treatment in this system.
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 treatment. 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
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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.

a more profound suppressive effect on B-cell activity. Concentrations that are inhibitory to tumor growth, there is likely to be

as with the drug-sensitive tumor, gemcitabine inhibited antibody formation (Fig. 7).

Mitogens (anti-CD3; anti-IgM; anti-CD28) were included to stimulate T-cell proliferation. Cell proliferation was assessed by 

in vitro 

antigens have demonstrated that these cells are the major effectors of antitumor immunity. 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

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

To further investigate the selective effect of gemcitabine on cellular and humoral immune responses, the IC50 was determined for different cell types. The IC50 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 IC50 for T cells was similar to the IC50 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.

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 [3H]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. 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 < 0.05; ** P < 0.001. This experiment was repeated 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 IC50 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 single 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 non-specific 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 toxoid 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 virus 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.
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