Cyclophosphamide induces type I interferon and augments the number of CD44hi T lymphocytes in mice: implications for strategies of chemoimmunotherapy of cancer

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Introduction

Cyclophosphamide (CTX) is a widely used chemotherapeutic agent in cancer therapy and in some autoimmune diseases. Combined regimens with CTX and immunotherapy are used in clinical trials with cancer patients. However, the mechanisms of CTX action are not fully understood. On the one hand, CTX can act as a conventional anticancer drug by affecting the in vivo proliferation of tumor cells. On the other hand, CTX can exhibit immunomodulatory effects, which may play an important role in the antitumor response.

In this regard, many studies have reported that CTX can increase the efficacy of immunotherapeutic agents by removing tumor-induced suppressor T cells. However, the nature of these suppressor cells is still a matter of debate.

By using transplantable mouse tumor models, we have recently reported that CTX can induce marked effects on T cells, which are important for a successful tumor eradication in response to adoptive immunotherapy. In particular, the results of an ensemble of experiments aimed at understanding the mechanisms underlying the synergistic antitumor response observed in tumor-bearing mice injected with CTX and tumor-sensitized lymphocytes have suggested that CTX acts by means of bystander effects (possibly through production of T-cell growth factors occurring during the rebound events after drug administration) that may sustain the proliferation, survival, and activity of the transferred lymphocytes.

In considering which CTX-induced factor(s) can be important for explaining the effects on T cells and the antitumor response observed in tumor models, we focused our attention on type I interferon (IFN). In fact, the working hypothesis that this cytokine could somehow induced in our experimental system with tumor-bearing mice. (2) Recent reports have clearly indicated that type I IFN is the major factor responsible for the in vivo proliferation and long-term survival of certain subsets of T cells (especially CD44hiCD8+ T lymphocytes) in response to viruses or other stimuli.

All of this prompted us to investigate whether the injection of CTX in normal mice could result in any induction of type I IFN and to characterize the effects of CTX on T cells.

The results reported in this article demonstrate that CTX can induce type I IFN expression, which may represent an important mediator of the immunomodulatory effects of CTX, especially with regard to the expansion and persistence of memory T cells.
**Materials and methods**

**Mice and in vivo treatments**

Six- to 8-week-old male DBA/2 mice were obtained from Charles River Breeding Laboratories (Calco, Italy). All mice were treated in accordance with the European Community Guidelines. For bromodeoxyuridine (BrdU)-incorporation studies, mice were given drinking water containing BrdU (Sigma Chemical, St Louis, MO) at 0.8 mg/mL, which was made fresh and changed daily. CTX (Sigma) was dissolved in 0.15 mol/L NaCl (saline) and filter sterilized, and 0.5 mL of freshly prepared solution was injected intraperitoneally (CTX-treated mice). Polyinosinic-polycytidylic acid (poly [I:C]) (Sigma Chemical, St Louis, MO) at 0.8 mg/mL, which was made fresh and quantitated by UV absorbance at 260 nm. One microgram of total RNA was incubated for 5 minutes with oligo-(dt) 12-18 (Pharmacia, Uppsala, Sweden) at 75°C, cooled at room temperature, and reverse-transcribed by 200 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Bethesda, MD) for 1 hour at 37°C in a final volume of 20 µL. We amplified 2 µL of complementary DNA in a final volume of 20 µL (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCI, 1.5 mmol/L MgCl2, 0.01% gelatin, 200 µmol/L deoxyribonucleoside triphosphate (dNTP), and 10 pmol of each primer) using a Perkin Elmer Thermal Cycler (Perkin Elmer, Mannheim, Germany), further purified, and then quantitated by UV absorbance at 260 nm. One microgram of total RNA was incubated for 5 minutes with oligo-(dt) 12-18 (Pharmacia, Uppsala, Sweden) at 75°C, cooled at room temperature, and reverse-transcribed by 200 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Bethesda, MD) for 1 hour at 37°C in a final volume of 20 µL. We amplified 2 µL of complementary DNA in a final volume of 20 µL (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCI, 1.5 mmol/L MgCl2, 0.01% gelatin, 200 µmol/L deoxyribonucleoside triphosphate (dNTP), and 10 pmol of each primer) using a Perkin Elmer Thermal Cycler (Perkin Elmer, Norwalk, CT). Samples were heated at 94°C for 5 minutes, and each cycle was performed as follows: 40 seconds denaturation at 94°C, 40 seconds annealing at 62°C, and 1 minute extension at 72°C. At the end, samples were further incubated at 72°C for an additional 10 minutes. Table 1 reports the cytokine primer sequences, the number of amplification cycles, and the size of the fragment amplified in this study. For reaction product visualization, 10 µL of each PCR product was run on 1% agarose gel in 0.5 µmol/L Tris-acetate buffer (pH 8.3) at 100 V. The gel was stained with ethidium bromide, and bands were visualized under ultraviolet light. The specificity of the amplified DNA was confirmed by Southern blotting. The efficiency of amplification was calculated by comparing the densities of the bands to a standard curve of known DNA amounts. Table 2 reports the amount of DNA in each sample.

**Preparation of spleen and lymph node cells suspensions**

Mice were killed and spleen and lymph nodes were removed aseptically. Lymph node cells were pooled from cervical, axillary, inguinal, and mesenteric nodes. Tissues were gently disaggregated with a tissue homogenizer and processed for a standard IFN assay on L929 mouse cell monolayers, as described in “Materials and methods.” Data represent the IFN titer detected in the serum from each individual mouse, expressed in experimental units. One of these units is the equivalent of 4 IFN reference units.

**Detection of murine α and β interferon messenger RNAs in the spleen by reverse transcriptase (RT–)polymerase chain reaction (PCR)**

At different times after treatments, mice were killed and their spleens were immediately removed and directly homogenized with a tissue homogenizer in 2mL RNAzol B (Bioteck, Houston, TX) in an ice bath. Total RNA was then subjected to chloroform extraction and isopropyl alcohol precipitation. RNA was resuspended in freshly prepared DNAse-free RNAse (Boehringer Mannheim, Germany), further purified, and then quantitated by UV absorbance at 260 nm. One microgram of total RNA was incubated for 5 minutes with oligo-(dt) 12-18 (Pharmacia, Uppsala, Sweden) at 75°C, cooled at room temperature, and reverse-transcribed by 200 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Bethesda, MD) for 1 hour at 37°C in a final volume of 20 µL. We amplified 2 µL of complementary DNA in a final volume of 20 µL (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCI, 1.5 mmol/L MgCl2, 0.01% gelatin, 200 µmol/L deoxyribonucleoside triphosphate (dNTP), and 10 pmol of each primer) using a Perkin Elmer Thermal Cycler (Perkin Elmer, Norwalk, CT). Samples were heated at 94°C for 5 minutes, and each cycle was performed as follows: 40 seconds denaturation at 94°C, 40 seconds annealing at 62°C, and 1 minute extension at 72°C. At the end, samples were further incubated at 72°C for an additional 10 minutes. Table 1 reports the cytokine primer sequences, the number of amplification cycles, and the size of the fragment amplified in this study. For reaction product visualization, 10 µL of each PCR product was run on 1% agarose gel in 0.5 × tris borate dectrophoresis (TBE) buffer. As molecular weight markers, 1 µg of 132 bp DNA ladder (GIBCO-BRL) were run in parallel. As positive controls for IFN-α or IFN-β RT-PCR, we used messenger RNA (mRNA)
extracted from IFN-α1-transduced CI-11 cells and IFN-β-transduced TSA/Cl-A4 cells, respectively. PCR products were visualized by means of ethidium bromide staining and UV transillumination. After electrophoresis, the relative density of mRNA bands stained with ethidium bromide was determined by LKB 2202 Ultrascan densitometer (Pharmacia, LKB, Uppsala, Sweden). Messenger RNA transcripts were expressed in absorbance units.

Statistical analyses

Data were analyzed by Student t test.

Results

Detection of interferon activity in sera of mice treated with CTX

In a first set of experiments, mice were injected intraperitoneally with 2 doses of CTX (83 or 150 mg/kg body weight), with saline or poly (I:C) (positive control for IFN production). Sera were collected at different times after injection and tested for IFN activity by a standard biological assay on L929 cells. Table 2 shows the IFN activity detected in sera from individual mice at 12, 24, and 48 hours post injection.
Kinetics of accumulation of IFN-α and IFN-β messenger RNAs in the spleen of mice treated with cyclophosphamide

We then evaluated the levels of type I IFN mRNA expression in splenocytes harvested at different times after CTX injection. Thus, total RNA was extracted from spleen cells of mice treated with saline, CTX (83 or 150 mg/kg), or poly (I:C) and processed for RT-PCR by using 2 sets of primers specific for mouse IFN-α1,2 and IFN-β, and for β actin as a control (Figure 1). Injection of Poly (I:C) caused a transient but marked increase in the expression of both mRNAs for IFN-α and IFN-β, compared with untreated control mice (Figure 1A). In CTX-treated mice, the peak of mRNA expression for both IFN-α and IFN-β was almost comparable to that obtained with poly (I:C), but the kinetics was quite different. As shown by densitometric analyses (Figure 1B and 1C), IFN-α and IFN-β mRNA expression in spleen cells from mice injected with poly (I:C) reached its maximum level 6 to 12 hours after treatment. In CTX-treated mice, the maximal expression for both IFN mRNAs peaked between 12 and 24 hours. The apparent delay in the induction of IFN mRNA expression in mice injected with the higher dose of CTX (especially observed for IFN-α) may be somehow dependent on a CTX-induced toxicity. In this regard, there was a consistent decrease in the spleen weight in CTX-treated mice; this appeared more marked in animals treated with the higher dose of CTX. (Figure 1D).

CTX injection results in the up-regulation of Ly-6C expression and bromodeoxyuridine incorporation in spleen lymphocytes

Ly-6C is a lymphocyte activation and differentiation antigen normally present on minor subsets of mature T cells, monocytes, macrophages, and endothelial cells. Previous studies have demonstrated that IFN-α/β is a cytokine capable of specifically enhancing Ly-6C expression. By cytofluorimetric analyses, we then evaluated the percentage of Ly-6C-positive T cells with respect to the number of total T lymphocytes in the spleens of mice treated with a single injection of CTX (83 mg/kg) 3, 6, 9, and 15 days before sacrifice. In the same experiment, spleen lymphocytes were also analyzed for BrdU uptake. As shown in Figure 2, there was a significant increase of Ly-6C-positive cells in CTX-treated mice in comparison with controls at 6 days after CTX treatment. Cell proliferation data, obtained by the BrdU-labeling technique, showed a substantial decrease in the percentage of proliferating cells at day 3 after CTX administration, followed by a significant and long-lasting increase at the subsequent time points. In a subsequent experiment, illustrated in Figure 3, we compared the Ly-6C expression in spleen lymphocytes at various times after treatment of mice with either poly (I:C) or CTX. Ly-6C expression was maximal in splenic lymphocytes 6 days after CTX treatment, whereas poly (I:C) induced a more intense but more transient Ly-6C antigen enhancement that peaked at day 3.

As shown in Figure 4, injection of mice with a potent preparation of anti–IFN-α/β antibodies resulted in a significant inhibition of the CTX-induced up-regulation of Ly-6C expression.
on spleen lymphocytes, suggesting that CTX-induced type I IFN was involved in enhancing the expression of this antigen.

**Increase of the number of CD44hi T lymphocytes in the spleen and lymph nodes of CTX-treated mice**

We then evaluated whether injection of CTX could affect the number of T cells in the spleen and lymph nodes at different times after injection. In general, no statistically significant differences were observed in the total number of CD4 or CD8 T lymphocytes recovered from the spleen or pooled lymph nodes of CTX-treated mice with respect to control injected mice on days 6 and 10, with the exception of a slight increase in the number of CD4 T cells detected in the spleen at 6 days after CTX injection (data not shown). Spleen or lymph node cells from mice treated with a single injection of CTX (83 mg/kg), poly (I:C), or saline were also labeled with biotinylated anti-CD44 and PE-conjugated anti-CD4 or anti-CD8 antibodies and assessed by cytofluorimetric analysis. As shown in Figure 5, there was a marked increase in the percentage of CD44hi T lymphocytes in both the spleen and the lymph nodes of CTX-injected mice, compared with saline-injected control animals. This increase reached its maximum level 10 days after treatment for both CD4 and CD8+ T-cell subsets. In poly (I:C)-injected mice, there was also a marked increase in CD44hi cell percentage, but the kinetics of this increase appeared to be slightly different in spleen cells as compared with CTX-treated animals.

![Figure 5. Increase in the percentage of CD44hi T lymphocytes in the CD4+ and CD8+ T cells from spleens and lymph nodes of mice treated with CTX or poly (I:C).](image)

**Discussion**

In this study, we have provided the first evidence indicating that CTX is an inducer of type I IFN expression in mice. The induction of type I IFN by CTX was demonstrated not only by the presence of biologically active IFN in the serum of the injected mice at 24 to 48 hours, but also by a progressive accumulation of mRNAs for IFN-α and IFN-β in the spleens, starting at 6 hours and lasting for at least 48 hours after injection. The comparison of the kinetics of induction by CTX with that observed after injection of poly (I:C) (a typical strong inducer of type I IFN in mice) revealed that the peak of IFN-β mRNA induction by poly (I:C) occurred earlier (at 6 hours), but appeared to decrease more rapidly than that observed after CTX injection. As for IFN-α mRNA induction, the kinetics of accumulation of IFN-α mRNA in mice injected with poly (I:C) was similar to that observed in mice treated with the lower dose of CTX (83 mg/kg), whereas a certain delay in the induction kinetics was detected in mice injected with the higher dose of CTX (150 mg/kg).

On the whole, these results indicate that CTX is an inducer of type I IFN at a time early after injection, before drug treatment might result in a strong myelosuppression27(Figure 7A) or in a detectable decrease in the spleen weight (Figure 1D), substantially before the rebound phenomenon, which starts 5 to 6 days after CTX injection.23 Thus, although the mechanisms of type I IFN induction by CTX remain uncertain, our results suggest that the type I IFN induction is a direct effect of the chemotherapeutic agent on some mouse cells and does not represent a secondary response to drug-induced myelosuppression or to cell-stimulation events occurring during the rebound phase. This CTX-induced IFN production appears to be capable of directly affecting some lymphocyte functions. In fact, CTX induced an up-regulation in the expression of Ly-6C antigen (a marker known to be specifically induced by type I IFN), and this induction was inhibited by injecting the mice with a potent preparation of anti–IFN-α/β antibodies. Of interest, CTX was also capable of inducing an enhancement of the percentage of proliferating (ie, BrdU+) spleen lymphocytes as well as of modulating the phenotype of T cells, especially by enhancing the number of CD4+ and CD8+ T cells exhibiting a memory (CD44hi) phenotype in both the spleens and the lymph nodes of the injected mice.

The notion that CTX can induce release of cytokines and growth factors during the rebound events after drug-induced immunosuppression is well documented.38,39 Some studies have recently suggested that CTX can induce a pattern shift of cytokines from Th2 to Th1 in tumor models several days after injection.40 However, our finding that CTX can induce an early and long-lasting expression of type I IFN suggests that this event can be directly important in mediating some of the immunomodulatory effects of CTX observed in a number of experimental systems.

CTX is a currently used drug in cancer therapy. Although CTX is generally considered to be a typical chemotherapeutic agent capable of directly inhibiting tumor cell proliferation, many studies have shown that CTX can also induce multiple immunomodulatory effects.7-20 In mouse tumor models, some groups had suggested that CTX can act by inhibiting T suppressor cells.21-25 However, the nature of these suppressor cells assumed to be inhibited by CTX has remained elusive.26 In a recent study, in which we have investigated the mechanisms underlying the impressive antitumor response in tumor-bearing mice subjected to a single injection of CTX followed by adoptive immunotherapy,27 we provided...
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