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Importance in timing of cyclophosphamide on the enhancement of interleukin-2-induced cytolysis*

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Summary. We investigated the in vivo effects of cyclophosphamide (CY) on interleukin-2(IL-2)-induced cytolytic function and spleen cell immunophenotype. Pretreatment of A/J mice with CY (25 mg/kg or 75 mg/kg) i.p. on days −10 and −15 followed by IL-2 (50000 U i.p. on days 0 to +3) resulted in increased lysis of YAC-1 target cells compared to the group receiving IL-2 without previous CY therapy. In contrast, when CY was given on day −5, the cytotoxicity against YAC-1 was not enhanced. Phenotypic analysis of splenocytes obtained from mice treated with CY on day −10 or −15 revealed a relative decrease in L3T4- and Lyt2-positive T cells. In vivo depletion of natural killer (NK) cells by anti-asialoG_{M1}, prior to IL-2 therapy, abrogated the enhancing effect of CY on cytolysis while in vivo elimination of T cells by anti-L3T4 and anti-Lyt2 monoclonal antibodies did not, indicating that in the absence of T cell antigenic challenge, the increased cytolytic function after CY administration is probably mediated through NK cells. These findings provide evidence that CY may be used more effectively in IL-2-based immunotherapy protocols, if consideration is given to timing of CY and IL-2 administration.

Key words: Cyclophosphamide – IL-2, NK activity – Cytolysis

Introduction

Cyclophosphamide (CY) has been utilized by many as an immunomodulatory agent against human and murine tumors. North et al. demonstrated that an important mechanism by which CY can increase the efficacy of immunotherapeutic regimens is by removal of tumor-induced suppressor T cells [2, 16]. Infusion of immune spleen cells into mice with methylcholanthrene-induced fibrosarcoma had no effect on tumor growth while CY alone had a temporary effect. In contrast, combination therapy with CY followed by immune spleen cells resulted in complete regression of tumors. Using different animal tumor models others have shown similar results [8, 10].

Berd et al. reported that CY pretreatment enhanced delayed-type hypersensitivity responses to melanoma and colorectal carcinoma tumor vaccines, with some melanoma patients showing significant antitumor responses [4, 5]. CY-induced depression of T suppressor cell activity was thought to be the important component in the enhancement of the immune response to tumor antigens. Others have demonstrated similar findings with renal cell carcinoma vaccine preceded by CY administration [22].

Using its effect on elimination of suppressor cells Rosenberg and others have utilized CY in both murine and human systems prior to tumor-infiltrating lymphocyte (TIL) therapy to provide an environment for optimal TIL response [6, 19–21]. Furthermore, Lafreniere showed that pretreatment of mice 1 day prior to spleen harvest increased expansion and cytotoxicity of TIL cultures [14]. CY has also been combined with interleukin-2 (IL-2) and has proved to be synergistic against a variety of murine tumor models [1, 7, 12, 17, 23].

Despite the extensive literature on the elimination of tumor-induced immune suppression and the synergistic effects of CY with cytokines in preclinical murine tumor models, very little is known on its effects on natural killer (NK) and lymphokine-activated killer activity [3, 13, 18]. In this report we demonstrate that CY can enhance the interleukin-2-induced cytolysis of YAC-1 cells if given at

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an appropriate time. These findings may be important for the development of more effective chemoimmunotherapy protocols.

Materials and methods

Mice. Female A/J mice (H-2^a), 6–10 weeks old, from Jackson Laboratories, Bar Harbor, ME., were used for the experiments. The animals were housed and fed ad libitum according to University of Minnesota Research Animal Resources guidelines.

Cyclophosphamide. Mice were given CY (Cytoxan; Mead Johnson, Evansville, Ind.) 25 mg/kg or 75 mg/kg. Vials containing 100 mg were diluted with sterile water to yield a concentration of 5 mg/ml and used within 1 h. Animals were injected intraperitoneally (i.p.) with 0.1 ml (0.5 mg), or 0.3 ml (1.5 mg) at various times prior to spleen harvest.

Interleukin-2. Recombinant human IL-2, with specific activity 1.5×10^7 U/mg, was provided by Hoffman LaRoche Inc. (Nutley, N. J.). Mice received 50000 U i.p. in 0.2 ml Hank's balanced salt solution (Gibco Grand Island, N. Y.) daily for 4 days at different intervals following CY.

Cell preparation and culture. After sacrificing the animals in a CO_2 gas chamber, the spleens were removed under sterile conditions and placed in tissue-culture medium (TCM) consisting of RPMI-1640 medium (Gibco, Grand Island, N. Y.) supplemented with 25 mM HEPES (Gibco), 2 mM L-glutamine (Gibco), 100 U/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco), 10 mM nonessential amino acids (Gibco), 1 mM sodium pyruvate (Gibco), 25 µM 2-mercaptoethanol (Sigma Chemical Company, St. Louis, Mo.) and 5% fetal bovine serum (Gibco). The spleens were then crushed with a glass bottle stopper and spleen fragments gathered in a pipette and filtered through a 100-gauge Nytex mesh (Tetko Inc., Elmsford, N. Y.). Splenocytes were washed once in TCM and separated over Ficoll/Hypaque. Interface mononuclear cells were washed three times and resuspended in fresh TCM.

Tumor target. YAC-1, a Moloney-murine-leukemia-virus-induced T cell lymphoma of A/Sn (H-2^a) origin (American Type Culture Collection, ATCC, Rockville, Md.) was maintained in culture for use in the cytotoxicity assays.

Estimation of cell number and viability. Fresh splenocytes and YAC-1 cells were counted with a standard hemocytometer after mixing 10 µl cell preparation with 10 µl trypan blue (Gibco).

Cytotoxicity assay. YAC-1 target cells [$(1-2) \times 10^6$ in 0.5 ml TCM] were incubated with 500 µCi $Na^{51}CrO_4$ (5000 µCi/ml), (Amersham Corporation, Arlington Heights, Ill.) for 1.5 h at 37° C. Targets were washed three times in cold TCM and resuspended at a concentration of 1×10^4 cells/ml. Samples of 50 µl (500 targets/well) were then added to 96-well V-bottomed microtiter plates (Nunc, Naperville, Ill.) into which effectors (fresh splenocytes) had been previously added in triplicate and serially diluted in TCM to yield effector-to-target ratios (E:T ratio) of 200:1, 100:1, 50:1, and 25:1. Spontaneous-release wells contained only TCM and ^{51}Cr-labelled targets; maximal-release wells contained detergent and ^{51}Cr-labelled targets. The microtiter plates were gently centrifuged at 100 *g* for 2 min and incubated at 37° C and 5% CO_2 for 4 h. The plates were then centrifuged at 200 *g* for 5 min after which 100 µl aliquots of supernatant were harvested into glass scintillation vials (Dynalon, Rochester, N. Y.) and 2.5 ml scintillation fluid was added (Cytoscint; ICN Biomedicals, Irvine, Calif.). Radioactivity was counted using an LKB 1216 liquid scintillation counter. Cytotoxicity was determined by the formula:

$$\text{cytotoxicity (\%)} = 100 \times \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}}$$

In each case the mean ^{51}Cr release (cpm) was used.

Table 1. Effect of cyclophosphamide (CY) followed by interleukin-2 (IL-2) on the immunophenotype and cytolytic function of fresh splenocytes[a]

CY	L3T4 (%)	Lyt2 (%)	L3T4+ Lyt2 (%)	Cytotoxicity YAC-1 (%)
No CY	28.3	11.8	40.1	21.6
CY 25 mg/kg				
Day 5	29.6	13.3	42.9	21.0
Day 10	14.5	5.7	20.2	43.8
Day 15	15.7	7.2	22.9	32.2
CY 75 mg/kg				
Day 5	31.6	14.1	45.7	14.8
Day 10	22.7	10.3	33.0	25.9
Day 15	20.4	8.4	28.8	35.3

[a] Mice received cyclophosphamide 0.5 mg (25 mg/kg) or 1.5 mg (75 mg/kg) 5, 10, or 15 days prior to a 4-day course of IL-2 50000 U i.p. daily. The cells were harvested and pooled (5 mice/group) 24 h following the last dose of IL-2. Fresh splenocytes were immunophenotyped using phycoerythrin(PE)-conjugated anti-L3T4 and fluorescein-isothiocyanate(FITC)-conjugated anti-Lyt2 as described in Materials and methods. Percentage cytotoxicity at 200:1 effector-to-target ratio against YAC-1 is shown. Data are from one of three similar experiments

Phenotypic analysis by immunofluorescence. Fresh splenocytes were washed in phosphate-buffered saline (PBS) containing 2% heat-inactivated fetal calf serum and 0.1% sodium azide (Sigma) and 5×10^5 cells were placed in 96-well round-bottomed microtiter plates. Monoclonal antibodies used included fluorescein-isothiocyanate(FITC)-conjugated anti-Lyt2 and phycoerythrin(PE)-conjugated anti-L3T4 (Becton Dickinson, Mountain View, Calif.). Double-color analysis was done by incubating saturating amounts of the FITC- and PE-conjugated monoclonal antibodies with the cells for 30 min at 4° C. The cells were then washed three times with PBS containing 2% fetal calf serum and 0.1% sodium azide and fixed with PBS containing 2% formaldehyde (Polysciences, Warrington, Pa.). Analysis was carried out using a Becton Dickinson FACScan.

Cell depletions. Hybridomas GK1.5 and 2.43 (American Type Culture Collection) were used to produce monoclonal antibodies for T cell depletions. Monoclonal antibodies were purified from ascites by ammonium sulfate precipitation, washed twice with 50% ammonium sulfate, and dialyzed against PBS. Mice received two 350 µg doses of GK1.5 (anti-L3T4) and of 2.43 (anti-Lyt2) antibody i.p. to deplete the $CD4^+$ and $CD8^+$ cells. Rabbit anti-(mouse asialoGM1) (Wako Chemicals, Richmond, Va.) has been previously described as useful for in vivo depletion of NK activity [9, 11, 15]. Two doses of anti-asialoGM1, 25 µl diluted in 175 µl PBS, were given i.p. to deplete mice of NK cells.

Statistical analysis. Student's *t*-test was used to compare cytolysis, number and immunophenotype of cells from groups of mice receiving or not receiving CY.

Results

In vivo effect of timing and dose of cyclophosphamide on IL-2-induced cytolysis of YAC-1 cells

Administration of 50000 U or 75000 U IL-2 i.p. for 4 days to A/J mice resulted in a similar increase in cytotoxicity of fresh splenocytes against YAC-1 target, from a baseline NK activity of 13.4% (at 200:1 E:T ratio) to 20.7% (50000 U) or 19.8% (75000 U). We therefore used

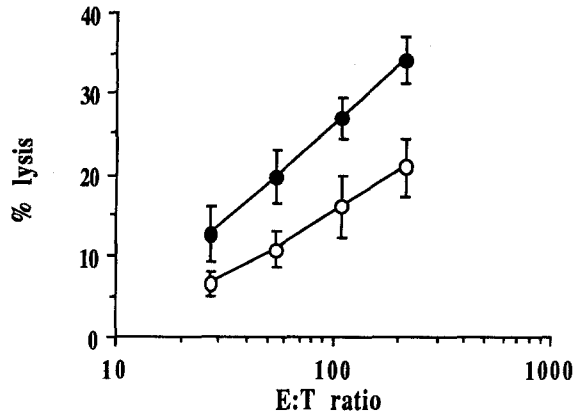

Fig. 1. Effect of cyclophosphamide on interleukin-2(IL-2)-induced cytolysis of YAC-1 cells. Mice received 25 mg/kg cyclophosphamide i.p. 10 days prior to a 4-day course of 50000 U IL-2 i.p. daily. The cytotoxicity of splenocytes from individual mice (six mice per group) was tested against YAC-1 targets 24 h following the last dose of IL-2 in 4-h ^{51}Cr-release assay using effector-to-target ratios of 200:1, 100:1, 50:1 and 25:1 (●). Results are compared to control mice receiving no CY prior to IL-2 (○). Student's *t*-test was used to compare cytolysis between the two groups at each E:T ratio (200:1 $P = 0.02$, 100:1 $P = 0.04$, 50:1 $P = 0.05$)

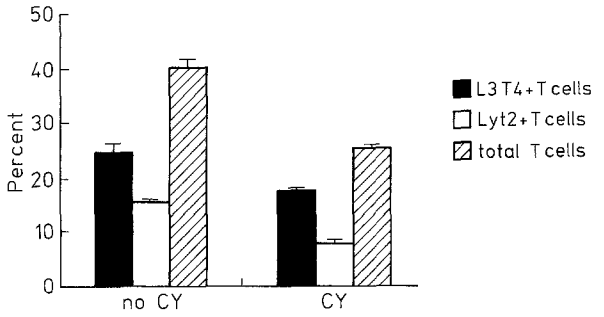

Fig. 2. Effect of cyclophosphamide followed by IL-2 on the immunophenotype of splenocytes. Mice received 25 mg/kg cyclophosphamide (CY) i.p. 10 days prior to a 4-day course of 50000 U IL-2 i.p. daily. The cells from six individual mice were harvested 24 h following the last dose of IL-2 and immunophenotyped using anti-L3T4-PE and anti-Lyt2-FITC. Results are compared to those from control mice receiving no CY prior to IL-2. Student's *t*-test was used to compare the percentage of T cell populations between the two groups (L3T4 $P = 0.001$, Lyt2 $P < 0.001$, total T cells $P < 0.001$)

50000 U IL-2 in experiments performed to evaluate the effect of CY on the cytolytic function of fresh splenocytes. Mice were pretreated with 0.5 mg CY (25 mg/kg) or 1.5 mg (75 mg/kg) i.p. 5, 10 or 15 days prior to receiving 50000 U IL-2 i.p. for 4 days. Cytotoxicity of pooled fresh splenocytes was tested against YAC-1 24 h following the last dose of IL-2, using a 4-h chromium-release assay (Table 1). The lower dose of CY (25 mg/kg) did not have any influence on lysis of YAC-1 while 75 mg/kg resulted in decreased cytolysis when given 5 days prior to initiation of IL-2 therapy. However, when CY (25 and 75 mg/kg) was administered either 10 days or 15 days prior to a 4-day course of IL-2, cytotoxicity against YAC-1 was augmented. The lower dose of CY (25 mg/kg) resulted in the highest cytotoxicity when administered 10 days prior to IL-2, while with 75 mg/kg, the lytic activity was higher when CY was given 15 days before IL-2. Using our optimal dose and timing of CY (25 mg/kg 10 days prior to IL-2), we examined the cytolytic function of splenocytes from individual mice. As shown in Fig. 1, CY significantly enhanced the IL-2-induced lysis of YAC-1.

In vivo effect of timing and dose of cyclophosphamide followed by IL-2 on immunophenotype of splenocytes

To determine if the changes in cytotoxicity were due to a preferential effect of CY on splenic cell subpopulations, splenocytes were immunophenotyped using anti-L3T4-PE and anti-Lyt2-FITC monoclonal antibodies. Administration of CY (25 and 75 mg/kg i.p.) on day –5 followed by IL-2 (50000 U i.p.) on days 0–3 resulted in equivalent or somewhat increased percentages of L3T4- and Lyt2-positive cells (Table 1). In contrast, earlier administration (day –10 or –15) decreased the proportion of T splenocytes. It therefore appears, that splenocyte populations with a higher proportion of T cells had lower cytolytic activity whereas those with a lower percentage of T cells had enhanced cytolysis on a per cell basis. In additional experiments (Fig. 2), administration of 25 mg/kg CY 10 days prior to IL-2 significantly decreased the L3T4$^+$, Lyt2$^+$ and total T cells compared to the group of mice receiving no CY. This dose of CY did not have an effect on the total number of cells per spleen with a mean ± SEM of 75.6±5.5 in the group receiving this agent compared to 73.3±12.6 in the group not receiving CY (*P*, NS) indicating that the absolute number of L3T4$^+$, Lyt2$^+$ and total T cells were selectively decreased in the CY group.

In vivo effect of cyclophosphamide on IL-2-induced cytolysis of YAC-1 cells by splenocytes of mice depleted of NK or T cells

To determine whether the enhancing effect of cyclophosphamide on cytolysis is mediated through NK or T cells, lytic function was assessed following in vivo depletion of these subpopulations. The lytic function of splenocytes from mice receiving CY (25 mg/kg) 10 days prior to IL-2 (50000 U i.p. ×4) and depleted of NK cells by i.p. administration of two 25-µl doses of anti-asialo-GM1 was compared to that of mice not receiving CY. Anti-asialoGM1 abrogated the augmenting effect of CY indicating that CY enhances lysis by increasing the number of NK cells in the spleen or by increasing their cytolytic function or both (Fig. 3). Mice were also depleted of T cells by administration of two doses of 350 µg anti-L3T4 and anti-Lyt2. This resulted in complete elimination of CD4$^+$ and CD8$^+$ cells as determined by two-color fluorescence-activated cell sorting (FACS) analysis. In contrast to NK depletion, elimination of T cells did not abrogate the enhancing effect of cyclophosphamide, confirming that this agent works through NK cells.

Discussion

The efficacy of CY as an immunomodulatory agent in tumor systems and its effects on suppressor T cells have

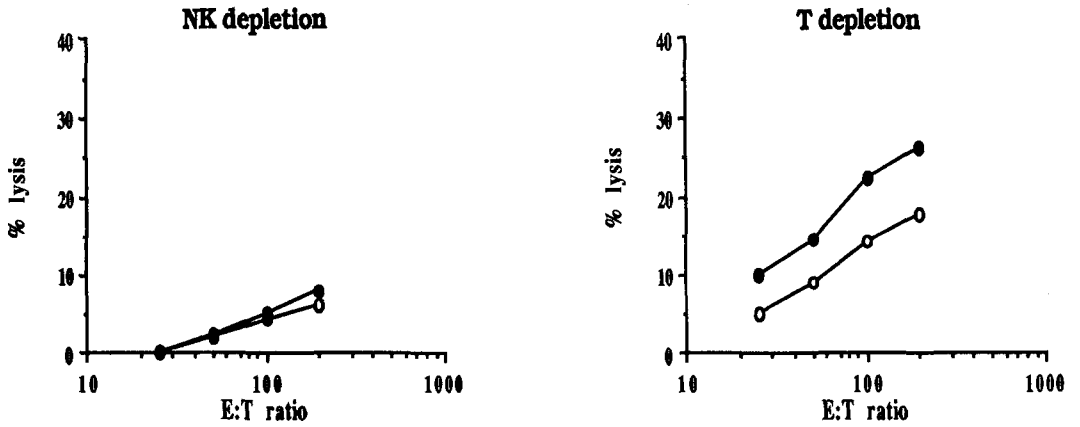

Fig. 3. Effect of cyclophosphamide on IL-2-induced cytolysis of YAC-1 cells by splenocytes of mice depleted of NK or T cells. Mice received 25 mg/kg cyclophosphamide (CY) i.p. 10 days prior to a 4-day course of 50000 U IL-2 i.p. daily. They received either 25 µl anti-asialoGM1 in 175 µl phosphate-buffered saline (*left*) or anti-L3T4 (GK1.5) and anti-Lyt2 (2.43), 350 µg of each (*right*), 2 days and 6 h prior to initiation of IL-2. The cytotoxicity of splenocytes (five mice per group) was tested against YAC-1 targets in a 4-h ^{51}Cr-release assay 24 h following the last dose of IL-2, using effector-to-target ratios of 200:1, 100:1, 50:1 and 25:1. Results are compared to control mice receiving no CY prior to IL-2. ●, with CY; ○, without CY

been examined by a number of investigators [2, 4–6, 8, 10, 14, 16, 19–22]. Others have shown that CY can be used successfully to potentiate the effect of IL-2 against murine tumor models. Kedar et al. reported that CY given 1–4 days prior to IL-2 was more effective than either agent alone in prolonging survival of mice with MCA-105 sarcoma [12]. Conversely, administration of CY following IL-2 therapy led to enhanced tumor growth and decreased survival. Similarly, Papa et al. reported that increasing doses of CY given 1 day prior to a 5-day course of IL-2 prolonged the survival of MCA-38- and MCA-105-bearing mice compared to that of mice treated with IL-2 or CY alone [17]. These investigators speculated that the synergistic therapeutic effects of CY may be due to direct tumor reduction by this chemotherapeutic agent, sublethal damage of tumor cells rendering them more susceptible to lysis by immune cells or elimination of suppressor cells. There is a relative lack of studies, however, examining the mechanisms of action of this drug on activated NK cytolytic function. Ricardi et al. reported that high-dose CY (240 mg/kg) resulted in depression of NK activity on day 4 with full recovery to levels of untreated controls 8 days following CY [18]. Ballas confirmed these findings by demonstrating that NK activity was absent 3 days and 6 days after CY treatment (300 mg/kg), but was fully restored and higher than the NK activity of normal spleens by day 12 [3]. We have shown that CY can be successfully combined with IL-2 to enhance lytic function. Timing of CY prior to administration of IL-2 was of utmost importance in augmenting cytotoxicity by fresh splenocytes. Both low-dose (25 mg/kg) and intermediate-dose (75 mg/kg) CY administered 10 days or 15 days prior to a 4-day course of IL-2 increased lysis of YAC-1 (Table 1). However, when CY was given 5 days prior to initiation of IL-2, the cytolysis of YAC-1 was not increased.

Stockman reported that 400 mg/kg CY resulted in a reduction of spleen weights to below 40% of the normal value within 24 h with some recovery by 7 days and an overshoot to nearly double the normal weight by 10 days [24]. Histological examination revealed that the B cell areas were almost totally depleted but the T cell areas remained largely unchanged. We have shown that a relative increase in L3T4 and Lyt2 cells occurred when CY (75 mg/kg) was given 5 days prior to a 4-day course of IL-2, with a decrease in the proportion of these subpopulations if CY (25 or 75 mg/kg) was given 10 days or 15 days previously (Table 1). It is evident from these data that cytotoxicity against the NK-sensitive target, YAC-1, was enhanced when the proportion of splenic T cells ($CD4^+$ and $CD8^+$) was depressed. We speculate that CY initially decreases the number of NK cells and that these cells increase by days 10–15 when T cells are relatively depressed. Administration of IL-2 at this time may allow a more pronounced activation of NK cells into cytolytic effectors. We have also demonstrated that depletion of NK cells but not T cells abrogated the enhancing effect of CY. This confirms that in non-tumor-bearing mice, in the absence of T cell antigenic challenge, the enhanced cytolysis following CY pretreatment is mediated through NK cells. CY may enhance lysis of YAC-1 by increasing the proportion of NK cells in the spleen or by directly or indirectly augmenting their cytolytic function. It has been suggested that CY decreases the suppressor subset of T cells [2, 8, 10, 16]. Therefore, decreased suppressor cell activity following CY may also play a role in enhancing effector function.

In summary, we have demonstrated that when CY is given at an appropriate dose and schedule, it acts synergistically with IL-2 to enhance cytolytic function. These findings provide evidence that this agent may be used more effectively in human chemoimmunotherapy protocols if consideration is given to the optimal timing of administration.

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