Augmentation of Human Natural Killer Cell Activity by Cyclophosphamide in Vitro

Basma Sharma and N. D. Vaziri

ABSTRACT

Cyclophosphamide (CY) has been shown to modulate a number of immune responses. In the present study, we have examined the effect of CY on human natural killer (NK) cells in vitro. Lymphocytes from six normal volunteers were cultured alone or with CY in the medium containing human AB plasma, and their cytotoxicity was assessed against cells of K562. The results demonstrated that lymphocytes when cultured with CY had 154 to 333% increase in NK cell activity as compared to lymphocytes that were cultured in the absence of CY. Similarly, CY markedly boosted the cytotoxic activity of lymphocytes from two healthy donors against cells from cultured B-leukemia 3163. Maximal augmentation occurred in cultured lymphocytes that expressed low levels of effector NK activity. Lymphocytes from 13 of 15 children with acute leukemia in remission when cultured alone had no reactivity to autologous leukemia cells. In the presence of CY, lymphocytes from eight of 15 of these patients developed cytotoxicity against autologous leukemia cells. CY was effective in augmenting NK cell activity at concentrations ranging from 7.5 to 60 μg/ml. Maximal potentiation of NK cell activity was induced after 8 hr of treatment with CY, although the increase in NK activity was started within 2 hr of incubation with CY. CY-activated NK cells were found in glass wool- and nylon wool-nonadherent cells. Treatment of lymphocytes with CY did not increase the extracellular levels of interferon. The results of this study demonstrate that CY can activate NK cells in vitro. The possible mechanisms of CY-induced augmentation of NK cell activity are discussed.

INTRODUCTION

CY has been used successfully as a cytotoxic agent in the treatment of certain neoplasms. It has also been used clinically in tissue transplantation and some autoimmune diseases (13-15, 23). In addition, CY has been shown to modulate immune responses in experimental animal models (1, 2, 4, 7, 17). Alterations in the immune responses appear to depend on the dose and schedule of administration by CY. Recently, mice with established tumors were cured when treated with a combination of CY and syngeneic immune lymphocytes (3, 9, 22).

We have reported recently that CY can induce modulation of human lymphocyte response to plant mitogens in vitro (19). It augmented the mitogenic response at lower concentrations, whereas it exerted a suppressive effect at higher concentrations. In order to understand whether the antitumor effect of CY is also mediated by simultaneous augmentation of NK cell activity, we examined in vitro the effect of CY on the human lymphocytes. The results described here demonstrate that CY can activate NK cells from an inactive stage to an active stage in vitro.

MATERIALS AND METHODS

Isolation of Lymphocytes. Mononuclear cells were isolated from heparinized peripheral blood of normal donors and leukemia patients over Ficol-Hypaque as described previously (18). Briefly, theuffy coat was removed and suspended in equal volumes of Hanks' balanced salt solution, layered over Ficol-Hypaque, and centrifuged at 500 x g for 20 min. The mononuclear cells recovered from the interface were washed 3 times and resuspended in MEM-15. Adherent cells were removed by serial passage of mononuclear cells through a glass wool column and then through a nylon wool column as described (12). T- and non-T-cells were separated by the erythrocyte rosette technique (10). Various cell fractions were resuspended in MEM-15, and their viability was determined by trypan blue exclusion. The viability of cells was over 90%. The percentage of monocyte-macrophages was determined by staining for α-naphthyl acetate esterase. Less than 2% of monocyte-macrophages was detected in nonadherent cells.

CY. CY was purchased from Mead Johnson Co., Evanville, IN. It was dissolved in distilled water and diluted to a concentration of 1 mg/ml with MEM before use.

Treatment of Lymphocytes with CY. Lymphocytes isolated from various donors were suspended in MEM-15 and then cultured alone or with various concentrations of CY at 37° in 5% CO2 humic atmosphere for 2 to 5 days. The cytotoxicity of treated and untreated cells was determined against tumor cells.

Titration of Interferon. Interferon was titrated in CY-treated or untreated lymphocyte culture supernatants by microassay as described previously (20). Briefly, a 0.05 ml of sample was delivered to the initial well of a microtiter plate, and 2-fold dilutions were carried out. A 0.05 ml of cell suspension containing 3 x 104 human muscle-skin fibroblasts was placed in each well, and the plate was incubated at 37° for 18 hr. The content of each well was removed, and then a 0.05-ml suspension containing 200 to 2000 TcLD50 vesicular stomatitis virus was added to each well. The plate was incubated at 37° for 14 to 24 hr. The interferon effect was scored in wells having 50% less degeneration than virus controls. One unit of interferon was defined as the amount that reduces the area of cytopathic degeneration by 50% compared with the corresponding controls not treated with interferon-containing samples.

Cell Lines. K562 cells were established from blast cells from a patient with chronic leukemia. Cultured malignant B-cells (3163) were provided by Dr. G. Granger, University of California, Irvine, CA. Cells were grown in MEM containing 10% fetal calf serum.

Cytotoxicity. The cytotoxicity of effector cells was determined by a 4-hr 51Cr release assay (18). Effector cells were incubated with 51Cr-labeled cryopreserved leukemia, K562, or 3163 target cells (107). After incubation, the tubes were centrifuged, supernatants were removed, and radioactivity was measured in a gamma counter. The percentage of 51Cr release was calculated.

1 This work was supported by USPHS Grant CA-27772 awarded by the National Cancer Institute, Department of Health and Human Services.

2 To whom requests for reprints should be addressed.

3 The abbreviations used are: CY, cyclophosphamide; NK, natural killer; MEM, minimal essential medium; MEM-15, complete minimal essential medium containing 20 μg 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 15% heat-inactivated AB plasma; ALC, autologous leukemia cells.

Received August 22, 1983; accepted April 30, 1984.
% of $^{51}$Cr release = $\frac{$^{51}$Cr release in $\frac{1}{2}$ supernatant}{$^{51}$Cr release in $\frac{1}{2}$ supernatant + $^{51}$Cr in residual} \times 100$

The specific release was determined, also, in the following way.

Specific release = $\frac{\text{Test release} - \text{spontaneous release}}{\text{Maximum release}} \times 100$

The test release is the mean of triplicate cpm released in the presence of effector cells, spontaneous release is the mean of triplicate cpm released by targets incubated alone, and maximum release is the mean of triplicate cpm released after lysis of targets by detergent. Experiments were carried out with several effector-target cell ratios, and activity was expressed in lytic units/10$^7$ cells, 1 lytic unit being defined as the number of cells required to produce 25% specific cytotoxicity.

Statistics. The statistical significance of CY effects was determined by a paired comparison $t$ test on differences of means.

RESULTS

Effect of CY on Lymphocyte Reactivity against Cryopreserved ALC. To determine whether CY can augment lymphocyte cytotoxic activity against ALC in vitro, lymphocytes from children with acute lymphoblastic and acute myeloblastic leukemia in remission were cultured alone or with CY. The cytotoxicity of fresh (spontaneous) and cultured effector cells was assessed against ALC. Fresh effector cells from 4 patients had spontaneous cytotoxicity against ALC greater than 10% (Table 1). When cultured alone in medium containing human AB plasma, lymphocytes from most of these patients lost all or most of their cytotoxicity. When cultured in the presence of CY, effector cells from 5 patients had significant enhancement ($P < 0.05$) in the cytotoxicity of ALC. CY also activated remission lymphocytes with no spontaneous cytotoxicity or that were cultured alone (Patients 4 to 8 and 15). It did not have any effect on remission lymphocytes with no activity from 5 of 15 patients. Lymphocytes from Patient 3 when cultured alone had no cytotoxicity in all experiments. Cytotoxicity against ALC was, however, developed in effector cells that were cultured in the presence of CY in all these 3 cases. Similar reproducible results were obtained with Patient 4.

Effect of CY on NK Cells. Lymphocytes from normal volunteers were cultured with or without the presence of CY to determine whether CY can modulate NK cell activity in vitro. The results given in Table 2 demonstrate that CY augmented the NK cell activity of 6 different donors against K562 cells. The increase in NK cell activity, as shown by the lytic units/10$^7$ cells, ranged from 154 to 333% of control. Maximum increases were seen in those healthy donors whose cultured effector cells had low NK activity. Similarly, treatment with CY boosted the cytotoxic activity of lymphocytes from 2 different donors against 3163 cultured B-leukemia cells (Table 3). CY was able to mediate augmentation of NK cell activity at concentrations ranging from 7.5 to 60 μg/ml. The representative experiment is shown in Chart 1. The maximum effect of CY was observed at concentrations of 7.5 to 15 μg/ml.

To study the kinetics of CY-mediated NK cell activation, lymphocytes from normal donors were cultured with CY for 2, 8, 16, and 40 hr, and after each incubation period, the cytotoxicity was assessed against K562. The maximum increase in NK cell activity was noted after 8 hr of treatment, although even after 2 hr of treatment with CY, an increase in NK activity was observed (Chart 2). Lymphocytes treated for 40 hr still had significantly greater cytotoxicity than did untreated cells.

Cellular Requirement for CY-mediated Augmentation. To determine whether monocyte-macrophages are essential for the CY-induced activation of NK cells, unfractionated mononuclear cells, before and after the depletion of glass wool- and nylon wool-adherent cells, were cultured with CY, and then their cytotoxic activity was assessed. Nonadherent mononuclear cells after treatment with CY demonstrated cytotoxicity against cultured B-cell leukemia, K562 cells, and cryopreserved ALC (Chart 3). The cytotoxicity of CY-treated nonadherent effector cells was higher than that of the untreated nonadherent effector cells. Cytotoxicity of cells in the erythrocyte-rosette fraction were also augmented by CY.

Antiviral Activity in CY-treated Lymphocyte Culture Supernatant. To determine whether CY-treated lymphocytes produce interferon and whether there is a correlation between CY-induced cytotoxicity and antiviral activity in CY-treated supernatant, lymphocytes from normal donors were cultured with CY for 2, 8, 16, and 40 hr, and after each incubation period, the antiviral activity was assessed. Nonadherent effector cells were cultured against ALC (Table 4). Lymphocytes treated for 40 hr still had significantly greater CPE-inhibitory activity than did untreated cells.

Statistics. The statistical significance of CY effects was determined by a paired comparison $t$ test on differences of means.
B. Sharma and N. D. Vaziri

Cytotoxic activity of CY-pretreated and untreated lymphocytes against B-leukemia cells at different effector:target ratios

<table>
<thead>
<tr>
<th>Donor</th>
<th>Treatment of effector cells</th>
<th>5:1</th>
<th>10:1</th>
<th>20:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>Untreated</td>
<td>1 ± 0.5</td>
<td>3 ± 0.5</td>
<td>4 ± 2</td>
</tr>
<tr>
<td></td>
<td>CY</td>
<td>3 ± 1</td>
<td>12 ± 0.9</td>
<td>19 ± 3.5</td>
</tr>
<tr>
<td>H</td>
<td>Untreated</td>
<td>1.5 ± 1</td>
<td>4 ± 1</td>
<td>6 ± 2</td>
</tr>
<tr>
<td></td>
<td>CY</td>
<td>4 ± 1</td>
<td>11 ± 2</td>
<td>18 ± 1</td>
</tr>
</tbody>
</table>

Table 3

DISCUSSION

The results presented here demonstrate that CY is capable of mediating augmentation of NK cell activity in vitro. These results confirm our previous findings that CY is active in vitro and can modulate lymphocyte response to plant mitogens (19). CY is able to increase NK cell activity against cultured K562 cells as well as against cultured B-leukemia cells. In addition, it can also augment the cytotoxicity of lymphocytes from children with acute leukemia in remission against cryopreserved ALC. The CY exerted maximum augmenting effect in the NK cells from donors whose lymphocytes cultured in the presence of human plasma showed little or no NK activity. Lymphocytes from normal donors showed little or no NK activity. Lymphocytes from normal donors whose lymphocytes cultured in the presence of human plasma at 37°C showed restoration of NK activity after 2 hr of in vitro incubation (Chart 2). A longer incubation period also resulted in the reduction of spontaneous cytotoxicity of fresh untreated and CY-treated effector cells, respectively.

Phenotypes were cultured alone or with CY. The treated and untreated cultured cells were tested for their cytotoxicity against K562 cells. The results show that CY-treated lymphocytes had much greater cytotoxicity than the cytotoxicity of untreated cells (Table 4). There was, however, no difference in antiviral activity of supernatants from lymphocytes cultured alone or with CY.

Table 4

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Lymphocyte culture</th>
<th>Interferon titer (units)</th>
<th>% of cytotoxicity of effectors against K562</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>UNF</td>
<td>&lt;12</td>
<td>14 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>UNF + CY</td>
<td>&lt;12</td>
<td>29 ± 3</td>
</tr>
<tr>
<td></td>
<td>GW-NAdC</td>
<td>24</td>
<td>28 ± 2</td>
</tr>
<tr>
<td></td>
<td>GW-NAdC + CY</td>
<td>24</td>
<td>38 ± 3</td>
</tr>
<tr>
<td>2</td>
<td>GW-NAdC</td>
<td>&lt;12</td>
<td>7 ± 1</td>
</tr>
<tr>
<td></td>
<td>GW-NAdC + CY</td>
<td>&lt;12</td>
<td>24 ± 2</td>
</tr>
</tbody>
</table>

<sup>a</sup> UNF, unfraccionated mononuclear cells; GW-NAdC, glass wool nonadherent cells.

Mean ± S.D.

Chart 3. Cellular requirement for the CY-mediated augmentation of NK cell activity. Unfraccionated mononuclear cells (UNF), nylon wool-nonadherent cells (NW-NAdC), glass wool-nonadherent cells (GW-NAdC), or erythrocyte-rosette-forming cells (ERFC) were cultured alone or with CY in MEM containing 15% human plasma at 37°C. The cytotoxicity of various effector cells was then assessed against K562 cells (A and B), 3163 (A and C), and cryopreserved human leukemia cells (C and D).
of treatment with 7.5 to 15 μg of CY/ml. Low-concentration CY also mediated potentiation of mitogenic responses (19). The higher concentrations were, however, usually suppressive. The differential dose-related influence of CY on mitogenic response and NK cells could be due to different target cells and via different mechanism(s).

Since CY was able to augment the NK cell activity of glass wool- and nylon wool-nonadherent cells, it appears that macrophages are not essential for the mediation of NK cell activation by CY. In fact, CY-induced augmentation was greater in glass wool- and nylon wool-nonadherent cells. The possibility that the residual macrophages may be involved is slim, as the mononuclear cells after passing through glass wool and nylon wool columns had less than 2% monocyte-macrophages.

The CY acts either directly on NK cells to potentiate their activity or stimulates the production of some soluble mediators that actually modulate the NK cell activity. Interferon and interleukin-2 both have been shown to stimulate NK cells (5, 6, 8, 11, 21, 24). Untreated and CY-treated culture supernatants had similar antiviral titers (<12 to 24 units), suggesting that interferon may not be involved in CY-induced augmentation of NK activity. The possibility that CY augments NK cell activity by increasing the intracellular levels of interferon and interleukin-2, however, cannot be ruled out. The CY-induced augmentation of lymphocyte-mediated cytotoxic activity may also be caused by CY action on immunoregulatory T-cells. When tested in vitro, CY had inhibitory activity on T-cells (19).

In conclusion, the results of this study demonstrate that CY can activate NK cells in vitro against cells of K562 and B-leukemia cell lines. It can also increase cytotoxicity of remission lymphocytes from some children with acute leukemia against ALC. These findings suggest that the antitumor effect of CY may also be attributed to its ability to mediate augmentation of NK cell activity in addition to its known direct cytotoxic effect on neoplastic and suppressor T-cells.

ACKNOWLEDGMENTS

The authors thank Dr. J. Tilles for determining antiviral activity in culture supernatants; Dr. G. Granger for providing B-leukemia cell lines; Dr. Sudhir Gupta for reviewing this paper; and Barry Siminina for excellent technical assistance. The excellent skill of Susan Burns in preparation of this manuscript is gratefully acknowledged.

REFERENCES

Augmentation of Human Natural Killer Cell Activity by Cyclophosphamide in Vitro

Brahma Sharma and N. D. Vaziri